EXHIBIT 13

PW 7021930

THE UNITED STATES OF AMERICA

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United States Patent and Trademark Office

August 07, 2006

THIS IS TO CERTIFY THAT ANNEXED IS A TRUE COPY FROM THE RECORDS OF THIS OFFICE OF THE FILE WRAPPER AND CONTENTS OF:

APPLICATION NUMBER: *09/063,551*
FILING DATE: *April 21, 1998*
PATENT NUMBER: *5,844,002*
ISSUE DATE: *December 01, 1998*

By Authority of the
Under Secretary of Commerce for Intellectual Property
and Director of the United States Patent and Trademark Office

H. L. Jackson

H. L. JACKSON
Certifying Officer

ISSUE CLASSIFICATION: Class 514 Subclass 649

[redacted]

PATENT NUMBER 5844002

DEC 01 1998

U.S. UTILITY PATENT APPLICATION

O.I.P.E.	PATENT DATE
SCANNED ____ Q.A. ____	

SECTOR	CLASS	SUBCLASS	ART UNIT	EXAMINER
	514	649	1614	Henley

FILED WITH: ☐ DISK (CRF) ☐ FICHE
(Attached in pocket on right inside flap)

PREPARED AND APPROVED FOR ISSUE

ISSUING CLASSIFICATION

ORIGINAL		CROSS REFERENCE(S)	
CLASS	SUBCLASS	CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)
INTERNATIONAL CLASSIFICATION			

☐ Continued on Issue Slip Inside File Jacket

☑ TERMINAL DISCLAIMER	DRAWINGS			CLAIMS ALLOWED	
	Sheets Drwg.	Figs. Drwg.	Print Fig.	Total Claims	Print Claim for O.G.
	None	None	None	10	1
☐ a) The term of this patent subsequent to ____ (date) has been disclaimed.	____ (Assistant Examiner) ____ (Date)			NOTICE OF ALLOWANCE MAILED: 6-24-98	
☑ b) The term of this patent shall not extend beyond the expiration date of U.S Patent. No. 5,362,755 5,547,994	RAYMOND HENLEY, III PRIMARY EXAMINER GROUP 1200 (Primary Examiner) (Date)			ISSUE FEE: Amount Due $1320.00	Date Paid 9/8/98
☐ c) The terminal ____ months of this patent have been disclaimed.	L.M. (Legal Instruments Examiner) 11-9-98 (Date)			ISSUE BATCH NUMBER: F87	

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Form PTO-436A
(Rev. 10/97)

(LABEL AREA) ISSUE FEE IN FILE

(FACE)

SERIAL NUMBER	FILING DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
09/063,551	04/21/98	514	1614	0701.027F

APPLICANT

TIMOTHY J. BARBERICH, CONCORD, MA; JAMES W. YOUNG, STILL RIVER, MA.

CONTINUING DOMESTIC DATA********

VERIFIED

THIS APPLN IS A CON OF 08/591,604 08/15/96 PAT 5,760,090
WHICH IS A CON OF 08/335,480 11/7/94 PAT 5,547,994
WHICH IS A CON OF 08/163,581 12/7/93 PAT 5,362,755
WHICH IS A CON OF 07/896,725 6/9/92 ABN
WHICH IS A CON OF 07/461,242 1/5/90 ABN

371 (NAT'L STAGE) DATA********

VERIFIED

MKA

FOREIGN APPLICATIONS********

VERIFIED

FOREIGN FILING LICENSE GRANTED 05/06/98

Foreign Priority claimed 35 USC 119 (a-d) conditions met	STATE OR COUNTRY	SHEETS DRAWING	TOTAL CLAIMS	INDEPENDENT CLAIMS
☐yes ☐no ☐yes ☐no ☐Met after Allowance Verified and Acknowledged _____ Examiner's Initials _____ Initials	MA	0	10	2

ADDRESS

PHILIP E HANSEN
HESLIN & ROTHENBERG
5 COLUMBIA CIRCLE
ALBANY NY 12203

TITLE

~~METHOD FOR TREATING ASTHMA USING OPTICALL PURE R(-) ALBUTEROL~~

Method for Inducing Bronchodilation Using Optically Pure R(-) Albuterol.

FILING FEE RECEIVED	FEES: Authority has been given in Paper No. _____ to charge/credit DEPOSIT ACCOUNT NO. _____ for the following:	
$395		☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time) ☐ 1.18 Fees (Issue) ☐ Other _____ ☐ Credit

PATENT APPLICATION SERIAL NO. _____

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE
FEE RECORD SHEET

04/24/1998 WILLARI 00000012 09063551
01 FC:201 395.00 OP

PTO-1556
(5/87)

ss Mail Label No. EL042[redacted]5US

EW UTILITY PATENT APPLICATION TRANSMITTAL
(Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No. 0701.027F

Total Pages in this Submission

TO THE ASSISTANT COMMISSIONER FOR PATENTS
Box Patent Application
Washington, D.C. 20231

Transmitted herewith for filing under 35 U.S.C. 111(a) and 37 C.F.R. 1.53(b) is a new utility patent application for an invention entitled:

METHOD FOR TREATING ASTHMA USING OPTICALLY PURE R(-) ALBUTEROL

and invented by:

Timothy J. Barberich and James W. Young

If a CONTINUATION APPLICATION, *check appropriate box and supply the requisite information:*

☒ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No.: 08/691,604

Enclosed are:

Application Elements

1. ☒ Filing fee as calculated and transmitted as described below
2. ☒ Specification having 10 pages and including the following:
   a. ☒ Descriptive Title of the Invention
   b. ☐ Cross References to Related Applications *(if applicable)*
   c. ☐ Statement Regarding Federally-sponsored Research/Development *(if applicable)*
   d. ☐ Reference to Microfiche Appendix *(if applicable)*
   e. ☒ Background of the Invention
   f. ☒ Brief Summary of the Invention
   g. ☐ Brief Description of the Drawings *(if drawings filed)*
   h. ☒ Detailed Description
   i. ☒ Claim(s) as Classified Below
   j. ☒ Abstract of the Disclosure
3. ☐ Drawing(s) *(when necessary as prescribed by 35 USC 113)*
   a. ☐ Formal
   b. ☐ Informal
   Number of Sheets _____

NEW UTILITY PATENT APPLICATION TRANSMITTAL (Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No. 0701.027F

Total Pages in this Submission

Application Elements (Continued)

4. ☒ Oath or Declaration
   a. ☐ Newly executed *(original or copy)* ☐ Unexecuted
   b. ☒ Copy from a prior application (37 CFR 1.63(d)) *(for continuation/divisional application only)*
   c. ☐ With Power of Attorney ☐ Without Power of Attorney

5. ☒ Incorporation By Reference *(usable if Box 4b is checked)*
   The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

6. ☐ Computer Program in Microfiche

7. ☐ Genetic Sequence Submission *(if applicable, all must be included)*
   a. ☐ Paper Copy
   b. ☐ Computer Readable Copy
   c. ☐ Statement Verifying Identical Paper and Computer Readable Copy

Accompanying Application Parts

8. ☐ Assignment Papers *(cover sheet & documents)*

9. ☐ 37 CFR(B) Statement *(when there is an assignee)*

10. ☐ English Translation Document *(if applicable)*

11. ☒ Information Disclosure Statement/PTO-1449 ☐ Copies of IDS Citations

12. ☒ Preliminary Amendment

13. ☒ Acknowledgment postcard

14. ☒ Certificate of Mailing
    ☐ First Class ☒ Express Mail *(Specify Label No.)*: EL042394445US

15. ☐ Certified Copy of Priority Document(s) *(if foreign priority is claimed)*

16. ☐ Small Entity Statement(s) - Specify Number of Statements Submitted: _____

NEW UTILITY PATENT APPLICATION TRANSMITTAL
(Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No. 0701.027F

Total Pages in this Submission

Accompanying Application Parts (Continued)

17. ☒ Additional Enclosures *(please identify below):*

Three (3) Terminal Disclaimers and fee ($165) therefor
Copy of Associate Power of Attorney

Fee Calculation and Transmittal

CLAIMS AS FILED

For	#Filed	#Allowed	#Extra	Rate	Fee
Total Claims	12	- 20 =	0	x $11.00	$0.00
Indep. Claims	3	- 3 =	0	x $41.00	$0.00
Multiple Dependent Claims (check if applicable) ☐					$0.00
				BASIC FEE	$395.00
OTHER FEE *(specify purpose)*					$0.00
				TOTAL FILING FEE	$395.00

☒ A check in the amount of $560.00 to cover the filing fee and Terminal Disclaimers is enclosed.
☒ The Commissioner is hereby authorized to charge and credit Deposit Account No. 08-1935 as described below. A duplicate copy of this sheet is enclosed.
- ☐ Charge the amount of as filing fee.
- ☒ Credit any overpayment.
- ☒ Charge any additional filing fees required under 37 C.F.R. 1.16 and 1.17.
- ☐ Charge the issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance, pursuant to 37 C.F.R. 1.311(b).

Dated: April 21, 1998

Signature

Philip E. Hansen
Reg. No. 32,700

Heslin & Rothenberg, P.C.
5 Columbia Circle
Albany, NY. 12203

ADDRESS ALL FUTURE CORRESPONDENCE TO:

PhilipE. Hansen
HESLIN & ROTHENBERG, P.C.
5 Columbia Circle
Albany, New York 12203

P01USMREV03

CERTIFICATE OF MAILING BY "EXPRESS MAIL" (37 CFR 1.10) Applicant(s): **Barberich et al.**			Docket No. **0701.027D**
Serial No.	Filing Date	Examiner	Group Art

Invention: **METHOD FOR TREATING ASTHMA USING OPTICALLY PURE R (-) ALBUTEROL**

I hereby certify that this **Patent Application**
(Identify type of correspondence)

is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 in an envelope addressed to: The Assistant Commissioner for Patents, Washington, D.C. 20231 on **April 21, 1998**
(Date)

Christine Kenedy
(Typed or Printed Name of Person Mailing Correspondence)

(Signature of Person Mailing Correspondence)

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("Express Mail" Mailing Label Number)

Note: Each paper must have its own certificate of mailing.

P06A/REV02

SPC89-05
1/4/90
PAC16

PATENT APPLICATION
DOCKET NO: SPC89-05

METHOD FOR TREATING ASTHMA USING OPTICALLY PURE R(-) ALBUTEROL

Description

Background

Albuterol is a drug belonging to the general class of beta-adrenergic compounds. The prime action of beta-adrenergic drugs is to stimulate adenyl cyclase, the enzyme which catalyzes the formation of cyclic-3',5'-adenosine monophosphate (AMP) from adenosine triphosphate (ATP). The cyclic AMP formed mediates the cellular responses. Albuterol acts selectively on beta$_2$-adrenergic receptors to relax smooth muscle tissue, for example, in the bronchial system. Albuterol is most commonly used to treat bronchial spasms associated with asthma and is the active component in well-known commercial bronchodilators such as Proventil and Ventolin.

The form in which albuterol is presently used is a racemic mixture. That is, it is a mixture of optical isomers, called enantiomers. Enantiomers are structurally identical compounds which differ only in that one isomer is a mirror image of the other and the mirror images cannot be superimposed. This phenomenon is known as chirality. Most biological molecules exist as enantiomers and exhibit chirality. Although structurally identical, enantiomers can have profoundly different effects in biological systems: one enantiomer may have a

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specific biological activity while the other enantiomer has no biological activity at all, or may have an entirely different form of biological activity.

Summary of the Invention

The present invention relates to a method of treating bronchial disorders, such as asthma, in an individual, by administering to the individual an amount of optically pure R(-) albuterol which is active in bronchial tissue sufficient to reduce bronchial spasms associated with asthma while minimizing side effects associated with albuterol. The method is particularly useful in treating asthma while reducing side effects, such as central nervous system stimulatory effects and cardiac arrythmia. In these applications, it is important to have a composition which is a potent broncho-dilator and which does not exhibit the adverse side effects of many beta-adrenergic drugs. A composition containing the pure R(-) isomer of albuterol is particularly useful for this application because this isomer exhibits these desired characteristics. The present method provides a safe, effective method for treating asthma while reducing undesirable side effects, for example, tremor, nervousness, shakiness, dizziness and increased appetite, and particularly, cardiac arrythmia, typically associated with beta-adrenergic drugs. In children, side effects such as excitement, nervousness and hyperkinesia are reduced when the pure isomer is

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administered. In addition to the above, at certain levels racemic albuterol can cause teratogenic effects, which are believed to be associated with the S(+) isomer. Administering the pure isomer reduces the teratogenic potential which is associated with the S(+) isomer of albuterol.

Detailed Description of the Invention

The present invention relies on the bronchodilation activity of the R(-) enantiomer of albuterol to provide relief from bronchial disorders, while simultaneously reducing undesirable side effects, for example, central nervous system stimulatory effects and cardiac disorders, commonly experienced by albuterol users. In the present method, the optically pure R(-) isomer of albuterol, which is substantially free of the S(+) enantiomer, is administered alone, or in combination with one or more other drug(s) in adjunctive treatment, to an individual in whom asthma relief (e.g., relief from bronchial spasms, shortness of breath) is desired. The optically pure R(-) isomer of albuterol as used herein refers to the levorotatory optically pure isomer of α^1[(tert-butylamino) methyl]-4-hydroxy-m-xylene-α, α'-diol, and to any biologically acceptable salt or ester thereof. The terms "optically pure" or "substantially free of the S(+) enantiomer" as used herein means that the composition contains at least 90% by weight of the R(-) isomer of albuterol and 10% by weight or less of the S(+) isomer. Optically pure albuterol is readily

obtainable by methods known to those of skill in the art, for example, by synthesis from an optically pure intermediate.

In the present method, the R(-) isomer of albuterol is administered to an individual who has asthma. For example, R(-) albuterol is administered to an individual after onset of asthma to reduce breathing difficulty resulting from asthma. In another embodiment, optically pure R(-) albuterol is administered prophylactically, that is, before the bronchiospasm begins in an asthma attack, to prevent its occurrence or to reduce the extent to which it occurs.

In the present method, R(-) albuterol can be administered by inhalation, by subcutaneous or other injection, orally, intravenously, topically, parenterally, transdermally, rectally or via an implanted reservoir containing the drug. The form in which the drug will be administered (e.g., inhalant, powder, tablet, capsule, solution, emulsion) will depend on the route by which it is administered. The quantity of the drug to be administered will be determined on an individual basis, and will be based at least in part on consideration of the individual's size, the severity of the symptoms to be treated and the result sought. In general, quantities of optically pure R(-) albuterol sufficient to reduce the symptoms of asthma will be administered. The actual dosage (quantity administered at a time) and the number of administrations per day will depend on the mode of

administration, for example, by inhaler, nebulizer or oral administration. About 30 mcg to about 90 mcg of the optically pure R(-) isomer of albuterol given by inhalation one or more times per day will be adequate in most individuals to produce the desired bronchodilation effect. For oral administration, e.g., tablet or syrup, a dose of about 1 mg to about 8 mg two to four times daily is administered to produce the desired effect.

In the method of the present invention, the optically pure R(-) isomer of albuterol can be administered together with one or more other drug(s). For example, an antiasthmatic drug such as theophylline or terbutaline, or an antihistamine or analgesic such as aspirin, acetaminophen or ibuprofen, can be given with or in close temporal proximity to administration of optically pure, R(-) albuterol. The two (or more) drugs (the optically pure active isomer of albuterol and another drug) can be administered in one composition or as two separate entities. For example, they can be administered in a single capsule, tablet, powder, or liquid, etc. or as individual compounds. The components included in a particular composition, in addition to optically pure albuterol and another drug or drugs, are determined primarily by the manner in which the composition is to be administered. For example, a composition to be administered in inhalent form can include, in addition to the drug(s), a liquid carrier and/or propellent. A composition to be administered in

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tablet form can include a filler (e.g., lactose), a binder (e.g., carboxymethyl cellulose, gum arabic, gelatin), an adjuvant, a flavoring agent, a coloring agent and a coating material (e.g., wax or a plasticizer). A composition to be administered in liquid form can include the combination of drugs and, optionally, an emulsifying agent, a flavoring agent and/or a coloring agent.

In general, according to the method of the present invention, the optically pure R(-) isomer of albuterol, alone or in combination with another drug(s), is administered to an individual periodically as necessary to reduce symptoms of asthma.

The present composition and method provide an effective treatment for asthma while minimizing the undesirable side effects associated with albuterol use. These side effects include central nervous system effects, such as tremor, nervousness, shakiness, dizziness and increased appetite, and cardiac effects, such as cardiac arrythmia. In children, side effects, such as excitement, nervousness and hyperkinesia, are reduced when the pure isomer is administered. In addition, teratogenic effects associated with albuterol are believed to reside in the S(+) enantiomer. Thus, administering the pure R(-) isomer may reduce the teratogenic potential associated with albuterol.

Equivalents

Those skilled in the art will recognize, or be able to ascertain, using no more than routine

experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed in the scope of the following claims.

CLAIMS

1. A method of treating asthma in an individual with albuterol, while reducing side effects associated with albuterol, comprising administering to the individual a quantity of an optically pure R(-) isomer of albuterol sufficient to result in bronchodilation, said R isomer being substantially free of its S(+) isomer.

2. A method of Claim 1 wherein the amount of the R(-) isomer of albuterol is greater than approximately 90% by weight.

3. A method of Claim 2 wherein the amount of the R(-) isomer of albuterol is greater than 99% by weight.

4. A method of Claim 1 comprising administering to the individual by inhalation from approximately 30 mcg to approximately 90 mcg of the R(-) isomer of albuterol per dose.

5. A method of Claim 1 comprising orally administering to the individual from approximately 1 mg to approximately 8 mg of the R(-) isomer of albuterol two to four times daily.

6. A method of treating asthma in an individual with albuterol, while reducing side effects associated with albuterol, comprising administering to the individual a quantity of an optically pure R(-) isomer of albuterol sufficient to result in bronchodilation and at least one additional drug.

7. A method of Claim 6 wherein the additional drug is selected from the group consisting of: bronchodilators, antihistamines and analgesics.

8. A method of Claim 7 wherein the analgesic is selected from the group consisting of: aspirin, acetaminophen and ibuprofen.

9. A composition comprising an optically pure R(-) isomer of albuterol and at least one additional drug.

10. A composition of Claim 9 containing at least 90% by weight of the R(-) isomer of albuterol.

11. A composition of Claim 10 containing at least 99% by weight of the R(-) isomer of albuterol.

12. A composition of Claim 9 wherein the additional drug is selected from the group consisting of: bronchodilators, antihistamines and analgesics.

08/063551

METHOD FOR TREATING ASTHMA USING OPTICALLY PURE R(-) ALBUTEROL

Abstract of the Disclosure

The optically pure R(-) isomer of albuterol, which is substantially free of the S(+) isomer, is a potent bronchodilator for relieving the symptoms associated with asthma in individuals. A method is disclosed utilizing the optically pure R(-) isomer of albuterol for treating asthma while minimizing the side effects associated with albuterol.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Declaration for Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

METHOD FOR TREATING ASTHMA USING OPTICALLY PURE R(-) ALBUTEROL

the specification of which (check one)

/ / is attached hereto.

/X/ was filed on January 5, 1990 as Application Serial No. 07/461,262 and was amended on ________ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)

(Number)	(Country)	(Day/Month/Year filed)	Priority Claimed	
			/ / Yes	/ / No
			/ / Yes	/ / No
			/ / Yes	/ / No

08471551.052498

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing date)	(Status, patented, pending, abandoned)
(Application Serial No.)	(Filing date)	(Status, patented, pending, abandoned)

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

I also hereby grant additional Powers of Attorney to the following attorney(s) and/or agent(s) to file and prosecute an international application under the Patent Cooperation Treaty based upon the above-identified application, including a power to meet all designated office requirements for designated states.

David E. Brook	Registration No. 22,592
James M. Smith	Registration No. 28,043
Leo R. Reynolds	Registration No. 20,884
Giulio A. DeConti, Jr.	Registration No. 31,503
Richard A. Wise	Registration No. 18,041
Patricia Granahan	Registration No. 32,227
Mary Lou Wakimura	Registration No. 31,804
Thomas O. Hoover	Registration No. 32,470
Paula A. Campbell	Registration No. 32,503
Alice C. Olek	Registration No. 33,542

all of Hamilton, Brook, Smith and Reynolds, P.C., Two Militia Drive, Lexington, Massachusetts 02173;

and

_____

_____

Send correspondence to: Patricia Granahan, Esq.
HAMILTON, BROOK, SMITH & REYNOLDS, P.C.
Two Militia Drive, Lexington, Massachusetts 02173

Direct telephone calls to: Patricia Granahan, Esq.
617-861-6240

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor Timothy J. Barberich

Inventor's Signature Timothy J. Barberich Date 2/28/90

Residence 73 Nashoba Road
Concord, Massachusetts 01742

Citizenship USA

Post Office Address SAME

Full name of second joint inventor, if any James W. Young

Second Inventor's Signature James W. Young Date 1 March 90

Residence 295 Still River Road
Still River, Massachusetts 01467

Citizenship USA

Post Office Address SAME

Full name of third joint inventor, if any

Third Inventor's Signature Date

Residence

Citizenship

Post Office Address

Full name of fourth joint inventor, if any

Fourth Inventor's Signature Date

Residence

Citizenship

Post Office Address

PATENT APPLICATION
Docket No. SPC89-05

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Timothy J. Barberich and James W. Young
Serial No.: 07/896,725 Group Art Unit: 1205
Filed: June 9, 1992 Examiner: L. Schenkman
For: METHOD FOR TREATING ASTHMA USING OPTICALLY PURE R(-) ALBUTEROL

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as First Class Mail in an envelope addressed to Honorable Commissioner of Patents and Trademarks, Washington, D.C. 20231 on July 14, 1993
HAMILTON, BROOK, SMITH & REYNOLDS, P.C.

B. J. Marini July 14, 1993
Signature Date

ASSOCIATE POWER OF ATTORNEY

The Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

The undersigned, as attorney of record, hereby grants to Philip E. Hansen, Registration No. 32,700, of the firm of Heslin & Rothenberg, 450 New Karner Road, P.O. Box 12695, Albany, New York 12212-2695, an Associate Power of Attorney in the above-captioned application.

Please continue to send all correspondence to the attention of the undersigned attorney at Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02173.

Respectfully submitted,

Patricia Granahan

Patricia Granahan
Registration No. 32,227
Attorney for Applicant(s)
(617) 861-6240

Lexington, Massachusetts
Dated: July 13, 1993

#112

Sheet 1 of 1

INFORMATION DISCLOSURE CITATION	Docket No. 0701.027D	Serial No. [redacted]
	Applicant: Barberich et al.	
	Filing Date:	Group:

U.S. PATENT DOCUMENTS

Examiner Initial		Document Number	Date	Name	Class	Subclass	Filing Date If Appropriate

FOREIGN PATENT DOCUMENTS

		DOCUMENT NUMBER	Date	Country	Class	Subclass	Translation	
							Yes	No
RA	BA	2 255 503	1992	UK			X	
RA	BC	DE2128258	1983	Germany				X
RA	BD	1298494	1971	UK			X	

Other Documents (including Author, Title, Date, pertinent public. etc.)

RA	CA	Tan et al. "Stereoselective Disposition of Salbutamol Enantiomers..." Clin. Chem. 33, 1026 (1987)
	CB	Brittain et al. "Some observations on the β-adrenoceptor agonist..." Br. J. Pharmac. 48, 144-147 (1973)
	CC	Hartley et al. "Absolute Configuration of the Optical Isomers of Salbutamol" J. Med. Chem. 12, 995 (1971)
	CD	Hawkins et al. "Relative Potency of (-)- and (±)-Salbutamol on Guinea Pig..." J. Med. Chem. 16, 856-857 (1973)
	CE	Buckner et al. "Studies on the Effects of Enantiomers of Soterenol, Trimetoquinol..." J. Pharm. Exp. Ther. 189, 616-625 (1974)
	CF	Passowicz-Muszynska E. "Effect on beta adrenergic receptors of tachyphylaxis..." Index Medicus 91:164287
	CG	Pauwels "Effect of corticosteroids on the action of sympathomimetics" Index Medicus 86:051970
	CH	Chapman et al. "An anomalous effect of salbutamol in sensitised guinea pigs" Brit. J. Pharmacol 99, 66P (1990)
	CI	Morley et al. "Effects of (+) and racemic salbutamol on airway responses in the guinea pig" Brit. J. Pharmacol. 104, 295P (1991)
	CJ	Chapman et al. "Racemic mixtures at root of worsening symptoms? Active enantiomers..." TIPS 13, 231-232 (1992)
RA	CK	Muittari et al. "Comparison of acute bronchodilator effects of oral salbutamol,..." Chem. Abstr. 89: 123259m (1978)

Examiner [signature] Date Considered 6/20/98

CF

(6)

3/5/4
07645287 91164287
[Effect on beta adrenergic receptors of tachyphylaxis on the sensitivity of smooth muscle in the bronchi to beta adrenergic receptor agonists in bronchial asthma]
WpLyw tachyfilaksji beta-adrenergicznych receptorow na wra.ANG.zliwosc miesni gLadkich oskrzeli na agoniste receptorow beta-adrenergicznych w dychawicy oskrzelowej.
Passowicz-Muszynska E
Katedry i Kliniki Chorob Wewnetrznych AM we WrocLawiu.
Pol Tyg Lek Jul 16-30 1990, 45 (29-31) p608-11, ISSN 0032-3756
Journal Code: PBY
Languages: POLISH Summary Languages: ENGLISH
Document type: JOURNAL ARTICLE English Abstract
JOURNAL ANNOUNCEMENT: 9106
Subfile: INDEX MEDICUS
The study involved 30 subjects: 15 healthy individuals and 15 patients with atopic bronchial asthma of the moderate degree. Salbutamol was administered to asthmatic patients in the intravenous infusion for 7 days. Beta-adrenergic receptor density in the lymphocytes and FEV1 were evaluated before and after therapy. Moreover, isoprenaline test was carried out to evaluate the sensitivity of the bronchial smooth muscle to beta-agonist. The test was performed prior to and after salbutamol therapy. It was found that beta-receptor agonist statistically significantly decreases beta-adrenergic receptor density. Equivalently, bronchial smooth muscle is less sensitive to beta-agonist in the same degree as a decrease in beta-adrenergic receptor density in the peripheral blood lymphocytes.
Tags: Female; Human; Male
Descriptors: *Albuterol--Therapeutic Use--TU; *Asthma--Drug Therapy--DT; *Bronchi--Drug Effects--DE; *Muscle, Smooth--Drug Effects--DE; *Receptors, Adrenergic, Beta--Drug Effects--DE; *Tachyphylaxis--Physiology--PH; Adolescence; Adult; Asthma--Physiopathology--PP; Lymphocytes--Drug Effects --DE
CAS Registry No.: 0 (Receptors, Adrenergic, Beta); 18559-94-9 (Albuterol)

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[Effect of corticosteroids on the action of sympathomimetics]
Influence des corticosteroides sur l'action des sympathicomimetiques.
Pauwels R
Bull Eur Physiopathol Respir Sep-Oct 1985, 21 (5) p53s-55s, ISSN 0195-3890 Journal Code: BGX
Languages: FRENCH Summary Languages: ENGLISH
Document type: JOURNAL ARTICLE; REVIEW English Abstract
JOURNAL ANNOUNCEMENT: 8603
Subfile: INDEX MEDICUS
Corticosteroids restore the bronchial responsiveness to beta-adrenergic stimulants in man. This has been shown both in severe asthmatic patients and in normal subjects, rendered insensitive by artificial means. On the contrary, in patients with bronchial asthma who have airways reactive to beta-adrenergic stimulants, the combination of corticosteroids and sympathicomimetics results in an additive effect of their bronchodilating capacity. Animal models, both in vivo and in vitro, show the same type of interaction between corticosteroids and beta-adrenergic stimulants. The mechanism by which corticosteroids restore the bronchial sensitivity to beta-adrenergic stimulation is not completely understood. Several mechanisms may be involved such as increased agonist binding, decreased receptor turn-over, increased uncoupling between receptor and adenylcyclase, decreased extraneuronal uptake, decreased COMT-activity. The relevance of the influence of corticosteroids on the metabolism of membrane phospholipids remains highly speculative. (15 Refs.)
Tags: Human
Descriptors: *Adrenal Cortex Hormones--Therapeutic Use--TU; *Adrenergic Beta Receptor Agonists--Therapeutic Use--TU; *Asthma--Drug Therapy--DT; Albuterol--Therapeutic Use--TU; Bronchodilator Agents--Therapeutic Use--TU; Drug Synergism; Drug Tolerance; Hydrocortisone--Therapeutic Use--TU; Isoproterenol--Therapeutic Use--TU; Methylprednisolone--Therapeutic Use--TU; Prednisolone--Therapeutic Use--TU; Pregnenediones--Therapeutic Use--TU; Tachyphylaxis; Terbutaline--Therapeutic Use--TU
CAS Registry No.: 0 (Adrenal Cortex Hormones); 18559-94-9 (Albuterol); 23031-25-6 (Terbutaline); 50-23-7 (Hydrocortisone); 50-24-8 (Prednisolone); 51333-22-3 (budesonide); 7683-59-2 (Isoproterenol); 83-43-2 (Methylprednisolone)

PERTUSSIS TOXIN INDUCES BRONCHOPULMONARY HYPERREACTIVITY TO HISTAMINE, 5-HT AND ANTIGEN IN GUINEA-PIGS, BUT SUPPRESSES RESPONSES TO FMLP

A. Imaizumi, J. Lefort & B.B. Vargaftig. Unité de Pharmacologie Cellulaire, Unité Associée Institut Pasteur-INSERM n° 285, 25 rue du Dr Roux, 75015, Paris, France.

Mice and rats inoculated with *Bordetella pertussis* vaccine show increased sensitivity to histamine, serotonin and anaphylaxis (Parfentjev and Goodline, 1948; Kind, 1958). This has been attributed to an acquired imbalance of two adrenergic effector systems, i.e., to a reduced functioning of the ß-adrenergic receptors or of some of the reactions between receptor activation and adrenergic end-response (Szentivanyi, 1968). We have shown that enhanced bronchoconstriction, BC (i.e., unspecific broncho-pulmonary hyperresponsiveness) follows the administration of a booster injection of antigen to actively sensitized guinea-pigs (Pretolani et al., 1988). This led us now to study the effects of pertussis toxin (PT), the active component of *B. pertussis* on broncho-pulmonary responsiveness. PT was administered i.v. to guinea-pigs at 0.8-20 µg/kg 6-72 h before they were stimulated, under pentobarbitone anesthesia, with i.v. histamine (0.5-16 µg/kg) or serotonin (0.5-8 µg/kg), at 10 min intervals. Bronchial resistance to inflation was evaluated by the method of Konzett-Rössler in cm H_2O. PT induced leukocytosis (lymphocytosis), and in 10 animals the number of circulating leukocytes increased from 5,700±800 to 38,900±3,700 at the dose of 20 µg/kg after 72 h. This effect was dose and time-dependent and started within 6 h. Initially no differences were observed between the bronchoconstrictor responses to histamine or to serotonin of control and PT-treated animals but, when propranolol was used (1 mg/kg i.v. and 3 mg/kg i.p.), BC was slightly increased only (% BC: 13.4±2.8 up to 19.6±3.5) in control, but was markedly increased (% BC; 8.9±2.8 to 70.8±4.4, p<0.001) in animals treated 72 h beforehand with PT at 20 µg/kg. Similar effects were observed with serotonin. In contrast, BC and the accompanying leukopenia induced by the i.v. administration of the secretagogue N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) (Boukili et al., 1988 and 1989) were antagonized by PT. Because of the contrasting effects on fMLP and on histamine and serotonin, isolated lungs provided by PT-treated animals where used . Under these conditions, BC and histamine and thromboxane A2 releases induced by the intra-pulmonary administration of fMLP were suppressed but the effects of OA (3 ng-100 µg injected to the lungs of guinea-pigs immunized with 10 µg ovalbumin (OA) in Al(OH)3 injected i.p. twice, at a 2-week interval) were enhanced. PT thus modifies negatively the signal transductions for cells involved in the lung responses to fMLP, but positively the effects of the direct constrictor agents histamine and serotonin and of antigen, which induces BC via these mediators. Our data suggest that PT prevents the effects of fMLP on a target other than the neutrophil, since it was effective on the isolated lungs (Boukili et al., 1989), possibly via its recognized effects on the Gi protein of other effector systems present in the lung. Hyperresponsiveness may result from an enhanced mediator release, possibly due to down regulation of a Gi protein, associated to a direct effect on smooth muscle, at a level which is under investigation.

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66P AN ANOMALOUS EFFECT OF SALBUTAMOL IN SENSITISED GUINEA-PIGS

I. Chapman*, L. Mazzoni & J. Morley, Preclinical Research, Sandoz Ltd., Basel CH-4002, Switzerland.

Eosinophils migrate to the intrapulmonary airways of sensitised guinea-pigs in response to inhaled allergen. Whilst assessing the capacity of anti-asthma drugs to inhibit this phenomenon, it was noted that animals pretreated with salbutamol (S) (1 mg/kg/day) by subcutaneous infusion invariably died on inhalation of allergen, in marked contrast to animals that were untreated or received other anti-asthma drugs. The contribution of altered airway smooth muscle function to this untoward effect has been investigated.

Guinea-pigs (450-600 gm) were sensitised by intraperitoneal injection (1 ml) of a suspension containing ovalbumin (OA, 10 ug/ml) and aluminium hydroxide (10 mg/ml) and separately with pertussis toxin (0.25 ml) on day 0, boosted on day 14 and implanted with either saline (C) or salbutamol (S) (1 mg/kg/day, Alzet minipump, s.c.) between day 21 and day 30. Six days later animals were anaesthetised with phenobarbitone (100 mg/kg i.p.) and pentobarbitone (30 mg/kg i.p.) paralysed with gallamine (10 mg/kg i.m.) and ventilated (1 Hz, 8 ml/kg) via a tracheal cannula. Airway resistance (R_L cm H_2O/l/sec) and compliance (C_{dyn} ml H_2O/l/sec) were calculated from measurement of tracheal airflow and transpulmonary pressure (Digital electronic pulmonary monitoring system, Mumed Ltd., U.K.). Animals were challenged with aerosolised OA (10-1000 ug/ml for 10 min) and changes in R_L and C_{dyn} were monitored at each breath. Airway responses to inhaled OA or intravenous histamine (1.0 & 1.8 ug/kg) were expressed as the maximal increase in R_L (mean±sem).
Responses to histamine in naive animals (107±67, 198±77, n=4) were not dissimilar from C animals (109±48, 252±91, n=10). Prior treatment with S (1 mg/kg/day s.c.) resulted in a slight reduction of these responses (46±12, 139±42, n=10, NS). No response to inhaled OA (100 ug) was observed in naive animals, in contrast to C animals (132±38, n=10) which developed increased reactivity to histamine following antigen challenge (418±64, 799±76, n=10). In animals pretreated with S, the reaction to antigen (334±58, n=10) was significantly (P<0.001) increased, even though airway responses to histamine were slightly reduced (225±66, 613±106, n=10).

The present results demonstrate that pretreatment of sensitised guinea-pigs with S augments the response to antigen. Altered distribution or increased dosage of inhaled allergen, altered airway reactivity or hypoxic vasoconstriction are mechanisms that might contribute to this phenomenon.

295P EFFECTS OF (+) AND RAC[illegible]IC SALBUTAMOL ON AIRWAY RESPONSES I[illegible]E GUINEA-PIG

J. Morley , I.D. Chapman, A. Foster, K. Hoshiko & L. Mazzoni. Preclinical Research, Sandoz Pharma Ltd., 4002 Basel, Switzerland.

In recent years, the incidence and severity of asthma, as well as associated death rates, have increased in several countries. It is appropriate therefore to ascertain whether anti-asthma drugs exhibit adverse effects that might contribute to these changes. An association between usage of beta-adrenoceptor agonist drugs and airway hyperreactivity in clinical asthma (Anonymous, 1990) has prompted study of (±)salbutamol, the most commonly used bronchodilator.

In the anaesthetised ventilated guinea-pig (Sanjar et al., 1990), reactivity of the airways to intravenous histamine (1.0-3.2 µg/kg) was enhanced significantly ($p < 0.01$, n=10,) following an intravenous infusion for one hour of (+)salbutamol (100 µg/kg), the non-bronchodilator enantiomer of racemic salbutamol. In studies with racemic salbutamol the bronchodilator action of (-) salbutamol precluded demonstration of airway hyperreactivity; hence, airway hyperreactivity was not detected following infusion of (±)salbutamol over 1 hour (100 µg/kg, n=10). However, increased responsivity to histamine was demonstrable four days after sustained subcutaneous infusion of (±)salbutamol (1 mg/kg/day, n=10), implying that the effect of (+)salbutamol on airway responsivity was less prone to tachyphylaxis than the spasmolytic effect of (-)salbutamol.

Subcutaneous infusion of (±)salbutamol (1 mg/kg) for more than two days increased the susceptibility of sensitised guinea-pigs to inhaled ovalbumin and caused almost 100 % mortality; an effect which was abrogated by inhalation of aerosolised (±)isoprenaline (0.1 % w/v) or subcutaneous injection of (±)salbutamol (1 mg/kg), immediately prior to inhalation of ovalbumin. Following subcutaneous infusion of (±)salbutamol (1 mg/kg, n=10) for 5 days, increased obstruction of the airways during inhalation or intravenous injection of ovalbumin was evident, which could account for death in such animals. Whether an increased incidence of neutrophils in the airway lumen observed 24 hours after inhalation of salbutamol (Boubekeur et al., 1989) contributed to the observed increase in airway reactivity has yet to be determined.

The capacity of (±)isoprenaline to induce airway hyperreactivity has been reported previously (Sanjar et al., 1990) and provides a plausible mechanism to account for the epidemic of asthma deaths twenty years ago (Speizer et al., 1968). In light of contemporary clinical evidence that bronchodilator therapy can be associated with enhanced airway reactivity, the pharmacology of (+)salbutamol and other (+)isomers of substituted catecholamines merits clinical investigation.

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Speizer, F.E., Doll, R. and Heaf, P. (1968) Br. Med. J., 1, 335-339.

296P NITRIC OXIDE AND ACETYLCHOLINE HYPERPOLARIZE SMOOTH MUSCLE CELLS IN THE RAT SMALL MESENTERIC ARTERY BY DIFFERENT MECHANISMS

C.J. Garland & G.A McPherson The Baker Medical Research Institute, Commercial Road, Prahran, Victoria 3181, Australia

Acetylcholine and related cholinomimetics stimulate endothelium-dependent hyperpolarization and relaxation in arterial smooth muscle cells (Bolton et al, 1984; Taylor & Weston, 1988; McPherson & Angus, 1991). The differential sensitivity of the hyperpolarization and relaxation to various blocking agents has led to the suggestion that these events are mediated by separate endothelium-derived factors (Taylor & Weston, 1988). Recently, Tare & co-workers (1990) have demonstrated that nitric oxide, which appears to be or is closely related to EDRF, can stimulate smooth muscle hyperpolarization as well as relaxation, implying a role for nitric oxide in the endothelium-dependent hyperpolarization to acetylcholine. The present study investigated and compared the responses to both acetylcholine and nitric oxide in the rat mesenteric artery in a myograph.

Smooth muscle cells in isolated segments of rat small mesenteric artery had a resting potential around -57mV. Both acetylcholine and nitric oxide stimulated concentration-dependent hyperpolarization. The hyperpolarization to acetylcholine was endothelium-dependent, and increased the membrane potential to around -67mV. If the artery was first exposed to noradrenaline (1-3µM), the smooth muscle cells contracted, and were depolarized to -35mV. Acetylcholine again hyperpolarized the membrane to around -67mV with the highest concentration tested (3µM) and in addition, reversed the contraction by over 90%. Both the hyperpolarization and the relaxation were unaffected by the presence of glibenclamide (3µM). Nitric oxide (0.1-1µmole), applied either as a gas in solution or released from acidified sodium nitrite, produced a transient hyperpolarization of the resting membrane potential which varied between 3 and 9mV. Unlike acetylcholine, the hyperpolarization was abolished by prior smooth muscle depolarization in the presence of noradrenaline, although at this time nitric oxide stimulated marked smooth muscle relaxation. Glibenclamide (3µM) reversably blocked the hyperpolarization of the resting membrane potential which occurred in response to nitric oxide.

These data show that the smooth muscle hyperpolarizations to acetylcholine and nitric oxide are induced in different ways. The voltage-dependent block of hyperpolarization to nitric oxide suggests the involvement of inwardly-rectifying potassium channels, which because of their sensitivity to glibenclamide may be ATP-dependent.

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TP Albuterol C.T.

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Racemic mixtures at root of worsening symptoms?

Active enantiomers may cause adverse effects in asthma

In a recent discussion in *TIPS*[1], of mechanisms whereby β_2-adrenoceptor-selective sympathomimetic drugs might worsen asthma symptoms, Barnes and Chung make no mention of the possibility that enantiomers of these racemic mixtures might be culpable. Isoprenaline, salbutamol, salmeterol and terbutaline have one chiral centre and are racemic mixtures of two enantiomers, with β_2-adrenoceptor agonist activity residing in the R-enantiomers. Fenoterol and formoterol have two chiral centres, giving rise to two possible diastereomers each having two enantiomers and, although marketed as single diastereomers, they are racemic mixtures of the R,R- and S,S-enantiomers.

Although it is generally accepted that the activity of a single enantiomer accounts for the biological effects of sympathomimetics, potent biological properties, unrelated to adrenoceptor occupancy, are documented. For instance, racemic tretoquinol not only relaxes airway smooth muscle but is also a potent inhibitor of platelet activation. Relaxation of guinea-pig trachea can be attributed to the (−)-S-enantiomer ($pD_2 = 7.10$) rather than the (+)-R-enantiomer ($pD_2 = 5.54$)[2], whereas inhibition of human platelet aggregation by the thromboxane A_2 mimetic U46619 is a property of (+)-R-tretoquinol ($IC_{50} = 0.99 \pm 0.02$ μM) rather than (−)-S-tetroquinol ($IC_{50} = 39.6 \pm 4.3$ μM)[3].

The capacity of sympathomimetics to facilitate sudden death in response to inhaled allergen or airway spasmogens in the guinea-pig is long established[4]. In studying the mechanism whereby salbutamol increases susceptibility of the sensitized guinea-pig to airway spasmogens[5], we noted that intravenous infusion of (+)-S-salbutamol induces airway hyperreactivity to leukotriene C_4 (Ref. 6) by a mechanism closely analogous

to that detailed for (+)-S-isoprenaline (i.e. unaffected by racemic propranolol but prevented by vagal section)[7].

More recently, we have observed that intratracheal instillation of S-isoprenaline, S-salbutamol and S-terbutaline are similarly efficacious in evoking increased airway responsivity to intravenous injection of histamine in the anaesthetized guinea-pig. Such observations demonstrate that enantiomers of sympathomimetics are not inert and hence may contribute to adverse effects of the type discussed by Barnes and Chung. It has long been recognized that use of sympathomimetics for asthma therapy is associated with a range of inconsistent, or frankly paradoxical, effects[8]. Rather than adding further material (i.e. glucocorticosteroids) to existing products as proposed, our findings indicate that it may be prudent to remove enantiomers that were previously thought to be biologically inert.

J. D. CHAPMAN, K. H. BUCHHEIT, P. MANLEY AND J. MORLEY

Preclinical Research, Sandoz Pharma Ltd, CH-4002 Basel, Switzerland.

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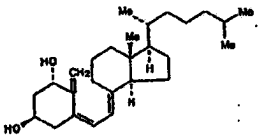
U46619: 9,11-dideoxy-11α-epoxymethano-prostaglandin $F_{2\alpha}$

TIPS 13 231-232 (1992)



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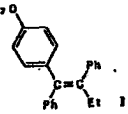
for 2 wk of *3-deoxy-1α-hydroxycholecalciferol* [53954-32-8] and 16 and 64 μg daily for 1 wk of *24R,25-dihydroxycholecalciferol* [55721-11-4] proved ineffective. In 32 successfully transplanted patients, restoration of normal or near normal renal function (serum creatinine <1.9 mg/100 mL) was not always followed by an immediate improvement in active Ca absorption. Ca absorption, esp. in female patients, was adversely affected by the required immunosuppressive *prednisone* [53-03-2] therapy and improvement was slow.

89: 123258k **Effect of tamoxifen pre-treatment on the retention of tritiated estradiol and 5α-dihydrotestosterone and on glucose metabolism in human breast carcinomas.** Deshpande, N.; Mitchell, Irene; Hughes, D. (Imp. Cancer Res. Fund, London, Engl.). *Eur. J. Cancer* 1978, 14(5), 473-7 (Eng). The effect of pretreatment with *tamoxifen* (I) [10540-29-1]



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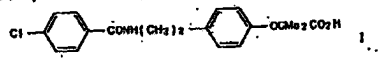
on *glucose* [50-99-7] metab. and the retention of injected *estradiol-17β* [50-28-2] and *5α-dihydrotestosterone* [521-18-6] by human breast carcinomas were studied in patients undergoing mastectomy. The pretreatment reduced retention of estradiol-17β, whereas a small but significant rise in 5α-dihydrotestosterone accumulation was obsd. There was an increase in both phosphofructokinase and glucose-6-phosphate dehydrogenase activities in tumors from treated patients, whereas α-glycerol-phosphate dehydrogenase activity was significantly reduced in the same tumors. These changes in carbohydrate metab. may not be due to the blocking of hormone receptors.

89: 123259m **Comparison of acute bronchodilator effects of oral salbutamol, salbutamol + hydroxyzine and ephedrine + theophylline + hydroxyzine combinations in asthmatic patients.** Muittari, A.; Ahonen, A. (Dep. Pulm. Dis., Tampere Cent. Hosp., Pikonlinna, Finland). *Curr. Ther. Res.* 1978, 23(5, Sect. 1), 567-74 (Eng). The bronchodilator effects of oral *salbutamol* [18559-94-9], the *hydroxyzine-salbutamol mixt.* [67650-17-3], and *ephedrine-hydroxyzine-theophylline mixt.* [65484-77-7] were obsd. in an acute study of asthmatic patients. All 3 drug combinations were able to increase peak expiratory flow (PEF) rates. The effect of oral salbutamol (4 mg) both alone and in combination with hydroxyzine (10 or 20 mg) was faster than the effect of the ephedrine-hydroxyzine-theophylline mixt., but the effect of the ephedrine-hydroxyzine-theophylline mixt. perhaps lasted longer. A second dose of the above ephedrine combination and the hydroxyzine-salbutamol mixt. given 5 h after the first dose was still able to increase the PEF rates. This was possibly an indication of the cumulative effect of the ephedrine-hydroxyzine-theophylline mixt., which was not so clearly seen with the salbutamol combinations. Otherwise, there were no differences between the effects or side effects of the drugs investigated in the present study. A combination of salbutamol and hydroxyzine seems, therefore, to be one rational means of treating asthma with fewer side effects than the salbutamol-hydroxyzine-theophylline mixt. but still about the same effectiveness. The dose of 10 mg of hydroxyzine had in combination about the same effect as 20 mg, but drowsiness was obsd. less frequently with the smaller dose.

89: 123260e **Changes in gastric secretion following administration of preparations affecting the parasympathetic and sympathetic nervous system.** Abasov, I. T.; Iof, I. M. (Azerbaijan Gastroenterol. Cent., Azerbaijan Res. Inst. Radiol. Oncol., Baku, USSR). *Acta Hepato-Gastroenterol.* 1978, 25(2), 144-9 (Eng). The changes in gastric secretion following administration of different prepns. was studied in a group of 180 patients with duodenal ulcer and chronic gastritis. In patients with hypersecretion the medication with *bishpan* [8075-97-6] and *Inderal* [3506-09-0], as compared with *atropine* [51-55-8], caused considerable shifts in the indices of gastric secretion. *Isoproterenol* [7683-59-2] exerted a stimulating influence both on initial hyper- and hyposecretion, whereas the other prepns. studied did not cause any essential rise in gastric secretion in patients with secretory deficiency. Reliable changes of the stimulated gastric secretion were obsd. only after Inderal. The data obtained can be useful when choosing a rational method for the therapy of gastric secretory disorders.

89: 123261f **The effect of bezafibrate on the fibrinolytic enzyme system and the drug interaction with racemic phenprocoumon.** Zimmermann, R.; Ehlers, W.; Walter, E.; Hoffrichter, A.; Lang, P. D.; Andrassy, K.; Schlierf, G. (Dep. Clin. Pharmacol., Med. Universitaetsklin. Heidelberg, Heidelberg, Ger.). *Atherosclerosis* (*Shannon, Irel.*) 1978, 29(4), 477-85 (Eng). *Bezafibrate* (I) [41859-67-0] given to patients with



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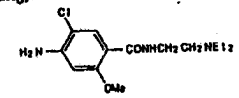
hyperlipemia on long-term treatment with racemic *phenprocoumon* [435-97-2] increased the anticoagulant response to the coumarin drug. Treatment with I at 450 and 600 mg daily required a redn. of the phenprocoumon dose by 18.5 and 33.5%, resp. Correspondingly, the serum level of phenoprocoumon decreased by 11.6 and 35.3%. No evidence for an altered drug elimination of racemic phenprocoumon was found during treatment with I. Apparently, I and analogous hypolipemic drugs enhance the response to oral anticoagulant drugs by increasing the affinity of the receptor site for coumarins or the rate of degrdn. of the vitamin-K-dependent clotting factors. The investigation of the fibrinolytic enzyme system demonstrated an increase of the fibrinolytic activity by enhancing the activity of the plasminogen activator. The lysis time for euglobulin clot was decreased significantly, plasma fibrinogen only moderately. The antiplasmin activity was not altered substantially by a decrease of *α1-antitrypsin* [9041-92-3] and a slight increase of α2-macroglobulin. In contrast with the inhibition of platelet function the effect of I on the fibrinolytic enzyme system showed no dose dependence.

89: 123262g **Influence of hyperprolactinemia due to metoclopramide on gonadal function in men.** Falaschi, P.; Frajese, G.; Sciarra, F.; Rocco, A.; Conti, C. (Ist. Patol. Med., Univ. Rome, Rome, Italy). *Clin. Endocrinol.* (*Oxford*) 1978, 8(5), 427-33 (Eng). In 5 male volunteers given *metoclopramide*



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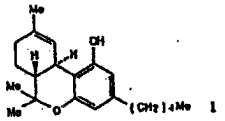
(I) [364-62-5] 10 mg three times daily for 6 wk, *prolactin* [9002-62-4], concns. were elevated in the blood. LH, FSH, testosterone and cortisol concns. were not altered. No change was obsd. in LH or FSH responses to LH-releasing hormone testing 4 wk after the beginning of therapy, compared with pretreatment values. A redn. in seminal vol. and total sperm count were obsd. in each subject. Four subjects noticed a decrease in libido and 3 lost spontaneous erections. While the I-induced hyperprolactinemia could be the cause of the obsd. changes in semen and erectile activity, this dopamine receptor blocking drug might directly affect central or peripheral mechanism of erection, the testis or accessory organs.

89: 123263h **Bronchodilator effect of Δ1-tetrahydrocannabinol.** Hartley, J. P. R.; Nogrady, S. G.; Seaton, A.; Graham, D. P. (Asthma Res. Unit, Sully Hosp., Penarth, Wales). *Br. J. Clin. Pharmacol.* 1978, 5(6), 523-5 (Eng). *Δ1-trans-Tetrahydrocannabinol* (I) [1972-08-3] produced bronchodilatation in asthmatic patients. Administered in 63 μL metered vols. contg. 50-200 μg by inhalation from an aerosol device to patients judged to be in a steady state, it increased peak expiratory flow rate and forced expiratory vol. in 1 s. The rate of onset, magnitude, and duration of the bronchodilator effect was dose related.



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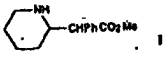
89: 123264j **Methylphenidate and serum prolactin in man.** Janowsky, David S.; Leichner, Pierre; Parker, Donal; Judd, Lewis; Huey, Leighton; Clopton, Paul (Dep. Psychiatry, Univ. California Med. Sch., La Jolla, Calif.). *Psychopharmacology* (*Berlin*) 1978, 58(1), 43-7 (Eng). In an expt. to evaluate the



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central dopaminergic effects of *methylphenidate* (I) [113-45-1], its effect on serum *prolactin* [9002-62-4] in psychiatric inpatients was examd. I did not exhibit a consistent effect on serum prolactin. Thus, its effect on serum prolactin does not parallel its behavioral activating properties, suggesting that such activation may not involve *dopamine* [51-61-6]. Possibly, norepinephrine or other noncatecholaminergic neurotransmitters are involved in I-induced behavioral activation.

89: 123265k **Physostigmine alters onset but not duration of REM sleep in man.** Gillin, J. Christian; Sitaram, N.; Mendelson, Wallace B.; Wyatt, Richard J. (Biol. Psychiatry

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710 MEASUREMENT OF SERUM FREE SALICYLATE LEVELS BY ULTRA-FILTRATION AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY, Tai C. Kwong, Joanne Fragnoli (Dept. Pathol. Lab. Med., U. Rochester Sch. Med. and Dent., Rochester, NY 14642)(Spon.: T.C.K.)

A procedure has been developed for measuring serum free salicylate levels by ultrafiltration in combination with a sensitive HPLC assay for salicylate.

Ultrafiltrate was obtained by centrifugation (2500 rpm, 10 min, room temp) of serum (1.0 ml) in an Amicon Centrifree microparitition device. Sample preparation for HPLC consists of mixing serum or ultrafiltrate with an equal volume of acetonitrile containing the internal standard 3, 4 dimethylbenzoic acid. Following vortex mixing (30s) and centrifugation (10000 rpm, 5 min.), 10 µl of the supernatant was injected into a liquid chromatograph equipped with a Varian 5µ, 4 x 15 cm C18 reversed phase column. The mobile phase, 24% acetonitrile in 60mM phosphoric acid, pH 2.3, was delivered at 1.8 ml/min. Chromatography was carried out at 45C and monitored at 237 nm.

Linearity of the calibration curve ranged from 1 to 1000 mg/L. Analytical recoveries at 50, 150 and 300 mg/l were greater than 97.5%. Acetylsalicylic acid and its metabolites as well as a large number of drug did not interfere. Patient results comparison between HPLC and a reference colorimetric assay (Kwong et al. Clin. Biochem. 17: 170, 1984) gave a correlation coefficient of 0.99 and a regression equation of Y(REF) = 0.91X(HPLC) + 14.9.

The assay precision for free salicylate at 8 and 57 mg/l was 10.2% and 6.8%, respectively. Ultrafiltration was validated by performing equilibrium dialysis on 31 patient specimens and comparing the free salicylate concentrations in the dialysates (X) with the free salicylate concentrations in the ultrafiltrates of the retentates (Y). The agreement was excellent with the regression equation of Y = 1.04X + 0.43, Sy.x = 2.33 mg/l and r = 0.998. Determination of free salicylate fraction in patient specimens by ultrafiltration and equilibrium dialysis confirmed the concentration-dependent protein binding of salicylate and the invalidity of equilibrium dialysis for free salicylate measurement.

(Ultrafiltration devices were a gift from Amicon)

711 SERUM PENTOBARBITAL DETERMINATION BY FLUORESCENCE POLARIZATION IMMUNOASSAY, Z. K. Shihabi and B. L. Smith (Dept. of Path., Wake Forest University, The Bowman Gray Sch. of Med., Winston-Salem, NC 27103) (Spon.: J. Jackson)

Pentobarbital is a short-acting, hypnotic barbiturate. It is administered in high doses to lower intracranial pressure in cerebral accidents; however, the serum level has to be regulated in a narrow range.

Recently a kit for qualitative detection of barbiturates in urine became available on the TDX (Abbott Laboratories, Irving, TX). Here we describe a simple modification to the kit for accurate determination of serum pentobarbital.

Procedure:

1. Calibrate the instrument according to the instructions of the manufacturer for general barbiturates.
2. Prepare a working standard of pentobarbital 50 mg/L in water.
3. Dilute the unknown patient, control and working standard 20-fold with the instrument buffer. (Samples should be free from phenobarbital.)
4. Assay according to the manufacturer's instructions.

Calculation (mg/L) = (Unknown reading/Standard reading) X50

Results:

The test is linear between 5-100 mg/L. The CV at 21 mg/L is 3.8% (n = 12). The linear regression of this method with the HPLC is y = 1.06x- 0.79, r = 0.99, n = 6). The average recovery of 3 spiked sera with 50 mg/L is 102%. Ten patients, free from barbiturates, yielded values below 1 mg/L by this method.

712 STEREOSELECTIVE DISPOSITION OF SALBUTAMOL ENANTIOMERS IN MAN: INVESTIGATION USING CHIRAL HPLC, Yok K. Tan and Steven J. Soldin (Dept. Clin. Biochem., Univ. Toronto, Res. Inst., Hosp. for Sick Children, Toronto, Ont. M5G 1X8) (Spon: J.G. Hill)

Salbutamol (albuterol) is used in asthma therapy as a racemic mixture of two stereoisomers, R(-)- and S(+)- salbutamol. The β-agonistic activity, however, resides mainly in the R(-)- enantiomer. We have investigated the stereoselective disposition of the two enantiomers in man after oral and intravenous administration of the racemic drug.

Enantiomers of salbutamol were successfully separated by HPLC with electrochemical detection on a chiral α_1-acid glycoprotein column. A specific urinary assay was then developed. The method requires sample clean-up procedures with an extraction efficiency of about 57% for the racemate. The inter-assay coefficients of variation for the determination of (-)- and (+)-salbutamol were 4.1 and 5.7%, respectively, at a drug level of 1.0 ng/ml. Concentrations and peak height ratios were linearly related over the concentration range examined (0.25-5.0 ng/ml).

Timed urine specimens collected from 1 subject after IV infusion of salbutamol racemate showed a continuously decreasing proportion of the active (-)-isomer. The excretion ratio of (-)-/(+)-isomer declined from 0.89 2 h after infusion to 0.58 after the 8-12 h period. Oral dosing on 3 subjects resulted in a similar excretion pattern but an even more extensive distortion in the enantiomeric ratio was revealed. This ratio can be increased to approach unity by arylsulfatase hydrolysis of the excreted salbutamol metabolites in acetate but not phosphate buffer.

We conclude that the renal excretion ratio of the enantiomers are not the same and are dependent on the route of administration. There is a stereoselective first-pass metabolism with the more potent (-)-isomer being preferentially conjugated to its sulfate ester. It is important to study concentration-effect relationship of salbutamol and possibly other β-agonists based on the measurement of the active enantiomer.

713 RAPID DETERMINATION OF SERUM ACETAMINOPHEN BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH A COLUMN SWITCHING TECHNIQUE - ON LINE PURIFICATION AND ANALYSIS -, Osamu Nozaki, T.Ohata, Y. Ohba (Dept. Clin. Path., Sch. Med., Univ. Kinki, Osaka 589, Japan) (Spon.: T.W. Wu)

We developed a rapid method for measuring serum acetaminophen by the reversed phase HPLC, which does not require previous sample purification. Our system consists of two columns, two HPLC pumps, a column switching valve, a UV monitor and a recorder. The first column is ϕ 4.6 x 30 mm, LC-SORB SPV ODS, 30 µm (Chemco, Japan) or ϕ 4.6 x 35 mm, BSA-ODS (TSK, Japan) and the second column is ϕ 4.6 x 50 mm, NUCLEOSIL ODS, 5 µm (M.Nagel, Germany). They are connected to the column switching valve (7000, Rheodyne, U.S.A.). We studied both types of first columns using two kinds of specimens (case 1: LC-SORB ODS/deproteinized serum, case 2: BSA-ODS/deproteinized serum and case 3: BSA-ODS/serum). Specimens (10-20 µl, containing internal standard of o-acetamidophenol) were injected into the first LC-SORB ODS column (mobile phase: 7 mM phosphate, flow rate 0.8 ml/min) or BSA-ODS column (mobile phase : 5 % methanol in 7 mM phosphate, flow rate 1.0 ml/min). The mobile phase of the second column was 10 % methanol in 7 mM phosphate (flow rate 1.0 ml/min). The column temperature was ambient. The UV wave length was 244 nm (UV monitor, HITACHI, Japan). The quantitation was evaluated by the peak height ratio between acetaminophen and I.S.. The retention time of acetaminophen was 5.58 (case 1), 7.58 (case 2), 6.50 (case 3) min. The intra- and inter- day coefficients of variation were under 3.0 % in all cases at 3, 30 µg/ml of acetaminophen. Recovery rates ranged from 94.1 - 109 % in all cases at 3, 30, 90 µg/ml of acetaminophen. Linearity of the peak height ratios versus acetaminophen concentrations was observed in the range of 0 - 120 µg/ml of acetaminophen.

Our method of acetaminophen determination by HPLC was rapid and accurate, and allowed omission of specimen pretreatment for HPLC and decreased specimen volume.

714 SERUM COCAINE CONCENTRATIONS BY RADIOIMMUNOASSAY AFTER INTRANASAL APPLICATION, A. Stewart, E.J. McQuay (Oxford Pain Relief Unit, Abingdon, Oxford, UK), C.W. Hand, Karen Ryan and R.A. Moore (DPC European Research Institute, Oxford OX8 6NN, UK). Spon: A. Paul Durham

Antiserum specific for cocaine (with low crossreactivity to benzoylecgonine, ecgonine methyl ester and ecgonine) was used with I-125 cocaine derivative to produce a 30-minute, double-antibody cocaine-assay in serum. To prevent the rapid breakdown of cocaine in serum, samples were taken into tubes containing sodium fluoride and potassium oxalate.

Patients undergoing oral surgery had intranasal cocaine to assist the passage of an intranasal catheter. Blood was taken between 5 min and 24 hr after the dose, and urine collections were made for at least 24 hr. Urinary benzoylecgonine was measured by DPC double-antibody Cocaine Metabolite kit.

Serum cocaine concentrations rose rapidly above 200 ng/ml within 5 min, and remained above 200 ng/ml until at least 3 hr. Significant serum cocaine concentrations (10 - 46 ng/ml) were found after 9 hr, and 3 patients had detectable levels (greater than 2.5 ng/ml) after 21 hr. Urinary benzoylecgonine levels were above 10,000 ng/ml in all patients 24 hr after the dose.

Br. J. Pharmac. (1973), 48, 144–147.

Some observations on the β-adrenoceptor agonist properties of the isomers of salbutamol

R. T. BRITTAIN, J. B. FARMER* AND R. J. MARSHALL†

Department of Pharmacology, Allen & Hanburys Limited, Ware, Herts.

Summary

1. The pharmacological activities of the optical isomers of salbutamol have been examined. (−)-Salbutamol was much more potent than (+)-salbutamol on β-adrenoceptors.

2. Both (−)- and (+)-salbutamol showed high selectivity for β-adrenoceptors in bronchial muscle compared to cardiac muscle, in this way resembling racemic salbutamol.

3. The use of isomeric activity ratio to detect differences between receptors was examined in the light of the results obtained with the isomers of salbutamol.

Introduction

Lands and his colleagues (Lands, Arnold, McAuliff, Luduena & Brown, 1967; Lands, Luduena & Buzzo, 1967) proposed that β-adrenoceptors be classified into β_1 and β_2 types. Stimulation of mammalian cardiac muscle is mediated by β_1-receptors and relaxation of bronchial, arterial and uterine muscle by β_2-receptors. Skeletal muscle also contains β_2-receptors. This classification, which was based on the relative potencies of N- and α-substituted catecholamines in different tissues, has gained more general acceptance since the discovery of highly selective β-adrenoceptor agonists (see Brittain, Jack & Ritchie, 1970). Salbutamol is a β-adrenoceptor agonist which is more active on bronchial smooth muscle than on cardiac muscle (Cullum, Farmer, Jack & Levy, 1969; Daly, Farmer & Levy, 1971). This drug contains an asymmetric centre and so it was of interest to ascertain whether activity resided mainly in the laevo (−) isomer (R configuration), as in the case with other sympathomimetic amines acting on adrenoceptors, and whether the isomers showed the same selectivity of action as the racemate. Recently Hartley & Middlemiss (1971) prepared the (−) and (+) isomers of salbutamol and this paper describes some pharmacological properties of these compounds.

Methods

Guinea-pigs of either sex, weighing 250–400 g were anaesthetized with urethane, 1·25 g/kg i.p., and prepared for measurement of bronchial resistance (Konzett & Rössler, 1940). Temporary increases in bronchial resistance, measured with a low pressure transducer connected to a Devices recorder, were produced by sub-maximal doses of acetylcholine, histamine or 5-hydroxytryptamine injected intravenously at intervals of 5 minutes. β-Adrenoceptor agonists were injected intravenously 5 min before intravenous injection of the spasmogens.

* Present address: Department of Pharmacology, Fisons Ltd., Loughborough, Leicestershire.
† Present address: University Department of Surgery, Western Infirmary, Glasgow, and Department of Pharmacology, University of Strathclyde, Glasgow.

...gle dogs of either sex, weighing 7–12 kg, were anaesthetized with pento... ...one sodium, 30 mg/kg intravenously. The animals were intubated with a ... endotracheal tube and allowed to breathe spontaneously, except in those ...iments involving pulmonary resistance tests. Arterial blood pressure was ...ured from a carotid artery or femoral artery by means of a Bell & Howell blood ...ure transducer connected to a Devices recorder. Heart rate was measured ... Devices instantaneous rate meter triggered by the pulse pressure. Measure... ...of pulmonary air flow, pressure and volume were made as described by ...nd (1967). Pulmonary resistance was calculated by the method of Amdur & ...(1958). Temporary changes in pulmonary resistance were induced by intra... ...injection of acetylcholine at 5 min intervals. β-Adrenoceptor agonists were ...1 min before the injection of acetylcholine. In all experiments drugs were ...ed intravenously through a cannula in a femoral vein.

...mulative concentration-effect curves for the β-adrenoceptor agonists were ...ined for the reduction of induced tone in the isolated intact trachea prepara... ...the guinea-pig (Farmer & Coleman, 1970) and for their positive inotropic ...hronotropic effects on isolated left and right atrial strips of the guinea-pig ...s, 1966) by adding geometrically increasing doses of drug to the tissue bath ...t changing the bathing fluid. The relative activities of the β-adrenoceptor ...ists were expressed as the doses equipotent with isoprenaline (=1) at 50% ...maximal effect.

...following drugs were used: (±)-isoprenaline sulphate, (±)-salbutamol hydro... ...ide, (−)- and (+)-salbutamol acetate monomethanolate, pronethalol hydro... ...ide and propranolol hydrochloride. (±)-Isoprenaline was dissolved in 0·9% ...NaCl solution (saline) containing ascorbic acid, 1 μg/ml. All other drugs ...dissolved in saline. In the text, concentrations refer to the free base; (±)- ...naline is referred to as isoprenaline.

Effects on bronchospasm in the anaesthetized guinea-pig

...renaline and (−)-, (+)- and (±)-salbutamol given intravenously inhibited ...holine-induced bronchospasm in anaesthetized guinea-pigs. The dose-effect ...for the isomers and (±)-salbutamol were similar in slope and maxima to ...obtained with isoprenaline. Effective intravenous doses were found in the ...of 1–10 μg/kg for isoprenaline, 2·5–100 μg/kg for (−)- and (±)-salbutamol ...5–5·0 mg/kg for (+)-salbutamol. The mean equipotent doses for (−)-, ...(±)-salbutamol compared to isoprenaline (=1) were 2·93 (1·30–6·57), 57 (57–233) and 3·75 (1·88–7·75) respectively. Similar orders of potencies ...ained when the spasmogen used was histamine or 5-hydroxytryptamine. ...cts of (−)- and (+)-salbutamol were mediated through β-adrenoceptors ...they were prevented by a prior injection of pronethalol, a β-adrenoceptor ...agent.

...ects on acetylcholine-enhanced pulmonary resistance and on blood pressure and heart rate in the anaesthetized dog

...aline, (−)-, (+)- and (±)-salbutamol caused dose-dependent inhibition ...se in pulmonary resistance induced by intravenous acetylcholine. The

effective intravenous dose-ranges were isoprenaline 0·1–2·0 μg/kg, (−)-salbutamol 1–4 μg/kg, (+)-salbutamol 50–400 μg/kg and (±)-salbutamol 4–20 μg/kg. The mean equipotent doses of (−)-, (+)- and (±)-salbutamol compared to isoprenaline (=1) were 2·6 (0·5–13·9), 138 (59–322) and 6·0 (3·0–12·2) respectively. Isoprenaline, (±)-salbutamol and the isomers, at the dose-ranges quoted, also caused falls in diastolic blood pressure (5–50 mmHg) but only isoprenaline caused significant increases in heart rate (10–60 beats/minute). Indeed very large doses of (−)- and (±)-salbutamol caused only small increases in heart rate; for example, the tachycardia after 100 μg/kg of either drug was only 20–25 beats/minute. (+)-Salbutamol, 400 μg/kg, had no significant effect on heart rate.

Effects on isolated tissue preparations

The effects of isoprenaline and (−)-, (+)- and (±)-salbutamol on isolated tracheal and atrial preparations of the guinea-pig are summarized in Table 1.

TABLE 1. *β-Adrenoceptor agonist activities of isoprenaline, (−)-, (+)- and (±)-salbutamol on isolated tissue preparations of the guinea-pig*

Preparation	Receptor type (Lands *et al.*, 1967)	β-Adrenoceptor agonist potency: (mean equipotent doses* relevant to isoprenaline=1)			
		Isoprenaline	(−)-Salbutamol	(+)-Salbutamol	(±)-Salbutamol
Intact trachea	β_2	1	6·2 (3·2–11·9)	425 (345–522)	5·6 (3·4–9·1)
Atria (left)	β_1	1	>10,000†	V. weak negative inotropic response	>10,000†
Atria (right)	β_1	1	>10,000†	>10,000†	>1,000†

* Calculated on weight/volume basis. † Partial agonist.

On the intact guinea-pig trachea the β-stimulants caused dose-dependent decreases in the rise of intraluminal pressure induced by transmural stimulation. The effective concentrations were: isoprenaline 5–50 ng/ml (12·9–129 nM), (−)- and (±)-salbutamol 20–300 ng/ml (84–1,260 nM) and (+)-salbutamol 1–35 μg/ml (4·20–105 μM). The concentration-effect curves for the isomers and (±)-salbutamol were similar in slope and maxima to those obtained with isoprenaline. The drug effects were antagonized by propranolol (100 ng/ml). The actions of (−)-, (+)- and (±)-salbutamol on isolated atrial preparations of the guinea-pig were quantitatively and qualitatively different from those of isoprenaline. Isoprenaline, 0·1–5 ng/ml (0·24–12·0 nM) caused dose-dependent positive chronotropic effects on cardiac muscle whereas concentrations of 0·2–20 μg/ml (0·84–84 μM) of (−)-, (+)- and (±)-salbutamol were required to elicit a positive chronotropic effect; even then the maximum responses to salbutamol were not more than 50% of those obtained with isoprenaline. In their inotropic actions (−)- and (±)-salbutamol were weak partial agonists; surprisingly (+)-salbutamol, 10–40 μg/ml (42–168 μM), caused a very weak negative inotropic effect (5–20%).

Discussion

Detailed studies with the isomer of isoprenaline (Beccari, Beretta & Laventalo 1953) showed that the pharmacological activity of the racemate resides mainly in the (−) isomer. It was not surprising therefore, to find that (−)-salbutamol was

more active than (+)-salbutamol. More interesting was the fact that both ers resembled the racemic compound in being very much more active on bronchial muscle than on cardiac muscle. This result makes it unnecessary ulate that the relative inactivity of racemic salbutamol on cardiac muscle s from an interaction between the isomers in the tissue.

(1969) has argued that if β-adrenoceptors are dissimilar, then the ratios of ty for the optical isomers of a β-adrenoceptor agonist in different tissues con- β-receptors should be different. Buckner & Patil (1971) determined iso- activity ratios of (−)- and (+)-isoprenaline in isolated atrial and tracheal rations of the guinea-pig and concluded that the β-adrenoceptors in these s were not different. While the isomeric activity ratio of (−)- and (+)-salbu- on isolated tracheal muscle is 1:63 it is impossible to calculate a ratio on ac muscle because the isomers are virtually inactive on this tissue. As dis- ed elsewhere (Brittain *et al.*, 1970) the selectivity of action of salbutamol ably stems from the nature of the N-substituent and the 3-substituent in the yl ring and not the configuration about the asymmetric carbon. The results pre- d in this paper are in accord with Lands' proposals that β-adrenoceptors in heal smooth muscle can be differentiated from those in cardiac muscle.

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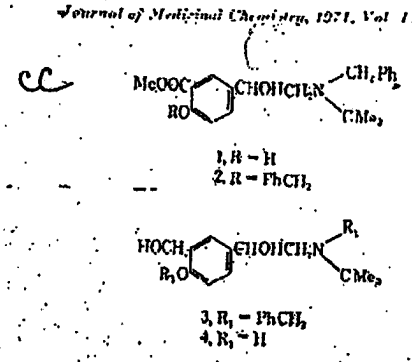
methoxyethylamine and ethyl acrylate using a 2-step procedure described by Bolyard and McElvain[9] for the synthesis of 1-arylalkyl-4-piperidones. The intermediate *N,N*-bis(β-carboxyethyl)-β-methoxyethylamine boiled at 111° (0.05 mm). The last 2 steps, the cyclization and decarboxylation, were done consecutively without isolation of the intermediate 3-carbethoxy-1-(2-methoxyethyl)-4-piperidone. The final product, a colorless liquid, boiled at 72–73° (0.5 mm). *Anal.* ($C_9H_{17}NO_2$) C, H, N.

α-[1-(1-Alkyl-4-piperidylamino)alkyl]benzyl Alcohols (3–11).—NaBH$_4$ (0.125 mole) was added in small portions over 2 hr to a stirred mixt of α-(1-aminoalkyl)-4-hydroxybenzyl alcohol HCl (0.025 mole), KOH (0.025 mole), and 1-alkyl-4-piperidone (0.177 mole) in 100 ml of MeOH. The reaction mixt was maintained at about 5° by means of an ice bath during the addn. After the addn the mixt was stirred for 1 hr at 20° and then acidified to pH 4 with 4 *N* HCl in EtOH. The mixt was filtered to remove ppted inorg salts and then evapd to an oil. The residual oil was triturated with boiling *i*-PrOH. The resulting white ppt was filtered off and recryst (see Table I).

α-[1-(4-Piperidylamino)alkyl]benzyl Alcohols (12 and 13).—A mixt of α-[(1-benzyl-4-piperidylamino)methyl]benzyl alcohol HCl (4 or 6) (0.055 mole) in 125 ml of aq 80% EtOH was hydrogenated for 8 hr at 3 atm using 5 g of 10% Pd/C as catalyst. The catalyst was removed by filtration, and the filtrate was evapd. The residue was recryst (Table I).

Pharmacology. Assay Method.—The test substances were injected in anesthetized dogs into the vascular bed supplied by the carotid artery. In this preparation a constant flow (peristaltic) blood pump was interposed between the proximal and distal segments of the right carotid artery. Prior to drug treatment, blood flow rate was set to result in a mean perfusion pressure approximately equal to systemic arterial blood pressure. Perfusion pressure was measured distal to the pump. Drug injections were made into the blood stream distal to the pump. Vasodilation was indicated by a decrease in perfusion pressure. Potency comparisons with papaverine were determined from plots of log molar dose *vs.* decrease in perfusion pressure. In this assay isoxsuprine was approximately 10 times more active than papaverine. The results reported in Table I are based upon the arbitrary assignment of papaverine potency at 100.

(9) W. W. Bolyard and S. M. McElvain, *J. Amer. Chem. Soc.*, 51, 922 (1929).



1, R = H
2, R = PhCH$_2$

3, R$_1$ = PhCH$_2$
4, R$_1$ = H

salt and the antipode was recovered from the mother liquors in good yield and reasonable optical purity.

Neutralization of the purified salts liberated the resolved bases 2 which on reduction with LAH followed by catalytic debenzylation gave the required isomers 4. The (+) and (−) forms were both characterized as their acetate salts.

The β-adrenoreceptive activities of these isomers were compared with those of the racemic compound on the β_1 receptors[4] of the isolated atria of the guinea pig and on the β_2 receptors[4] of the intact trachea of the guinea pig (Table I). In the latter test[5] the (−) isomer was approximately equiactive with the racemate and 80 times more potent than the (+) isomer. A similar pattern was shown in the much weaker effects on the force of contraction of the electrically driven left atrium.[6] However, both isomers were much less active than the racemate in increasing the rate of contraction of the spontaneously beating right atrium.[7] Although this result has been verified on several occasions, the very low order of activity precludes any useful interpretation of the apparent synergism in the racemate.[8]

TABLE I

BIOLOGICAL ACTIVITY OF THE ENANTIOMERS OF SALBUTAMOL

Compound	Biological test[a]		
	Trachea (β_2)	Left atrium (β_1)	Right atrium (β_1)
Racemate	4.3	15,000[b]	1,000
(−) Isomer	6.6	15,000[b]	10,000[b]
(+) Isomer	523	Inactive	100,000[b]

[a] These results represent the ratio of the amount of drug required to produce an equivalent response to a unit of isoprenaline. [b] Denotes partial agonist activity.

Comparison of the CD spectra of the salbutamol isomers with that of (*R*)-(−)-octopamine[9] showed that (−)-salbutamol had the *R* configuration. Both levorotatory compounds showed a clear negative Cotton effect at 276–280 nm. At lower wavelengths, 220–230 nm, the curves tended toward a further negative peak although this is somewhat masked by the high aromatic absorption.

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Absolute Configuration of the Optical Isomers of Salbutamol

D. HARTLEY* AND D. MIDDLEMISS

Chemistry Department, Allen and Hanburys Ltd., Ware, Hertfordshire, England

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The synthesis and biological properties of the β-adrenomimetic amine, salbutamol (4), have been described.[2] This compound is more selective for β_2 receptors than previously known drugs and therefore it was of interest to test each enantiomer to ascertain if the activity resided mainly in the (*R*)-(−) configuration as is the case with other drugs which act at adrenoreceptors.

Attempted resolutions of salbutamol or any phenolic precursor were unsuccessful.[3] However, when the phenolic group of the intermediate ester 1 was protected as the benzyl ether 2 then resolution was efficiently achieved with either (+)- or (−)-di-*p*-toluoyltartaric acid. In each case only one isomer formed a crystalline

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envelope and OH), 3.75 (s over a multiplet, 2, $ArCH_2$), 7.47 (s, 5, aromatic). The hydrochloride was prepared in the normal manner and recrystallized from EtOH–Et_2O, mp 203–205°. *Anal.* ($C_{14}H_{20}ClNO$) C, H, N.

Further elution with petroleum ether–Et_2O (1:1) afforded the trans alcohol (0.3 g): mp 75–76°; ir (0.002 *M* CCl_4) 3620 cm^{-1} (free OH); nmr (CDCl$_3$) δ 1.1–2.2 (broad signals, 6, bicyclic envelope), 2.2–3.0 (broad signals, 4, H-1, H-3, and H-4), 3.75 (s, 2, NCH_2Ar), 4.0–4.4 (m, 1, H-6), 7.45 (s, 5, aromatic). *Anal.* ($C_{14}H_{19}NO$) C, H, N.

6-trans-Hydroxy-2-azabicyclo[2.2.2]octane. A solution of 2-benzyl-6-*trans*-hydroxy-2-azabicyclo[2.2.2]octane (13.0 g, 0.06 mol) in EtOH (100 ml) was hydrogenated (3.15 kg/cm^2) over 10% Pd/C (1.5 g) for 24 hr. The catalyst was removed by filtration and the solvent evaporated to yield a white solid. Recrystallization from $CHCl_3$–hexane gave 6.0 g (78%) of a white solid: mp 229–231°; ir (1% $CHCl_3$) 3620 (OH), 3350 cm^{-1} (NH); nmr (DMSO-d_6) δ 1.0–2.4 (broad signals, 9, bicyclic envelope), 2.5–2.75 (m, 1, H-1), 2.8 (broad singlet, 1, NH), 3.4 (broad singlet, 1, OH), 3.75–4.2 (m, 1, H-6). *Anal.* ($C_7H_{13}NO$) C, H, N.

2-Methyl-6-*trans* hydroxy-2-azabicyclo[2.2.2]octane (7). A solution of 6-*trans*-hydroxy-2-azabicyclo[2.2.2]octane (5.0 g, 0.04 mol) and CH_2O (5 ml of 37%) in EtOH (70 ml) was hydrogenated (3.15 kg/cm^2) over 10% Pd/C (0.3 g) for 12 hr. The catalyst was removed by filtration and the solvent evaporated to yield a yellow oil which upon distillation gave 7 (4.5 g, 80%): bp 125–128° (20 mm); picrate mp 230–231°; ir (CCl_4, 0.002 m) 3640 cm^{-1}. This alcohol is identical with the alcohol obtained in Scheme II.

6-cis-Hydroxy-2-azabicyclo[2.2.2]octane. A solution of 2-benzyl-6-*cis*-hydroxy-2-azabicyclo[2.2.2]octane (1.7 g, 0.008 mol) in EtOH (80 ml) was hydrogenated (3.15 kg/cm^2) over 10% Pd/C (0.2 g) for 3 hr. The catalyst was removed by filtration and the EtOH evaporated. The crude product was recrystallized from Et_2O to yield a white solid (0.5 g, 50%): mp 193–195° dec; ir (1% $CHCl_3$) 3645, 3620, and 3380 cm^{-1} (OH and NH).

2-Methyl-6-*cis*-hydroxy-2-azabicyclo[2.2.2]octane (5). A solution of 6-*cis*-hydroxy-2-azabicyclo[2.2.2]octane (1.0 g, 0.008 mol) and CH_2O (1 ml of 37%) in EtOH (50 ml) was hydrogenated (3.15 kg/cm^2) over 10% Pd/C (0.2 g) for 6 hr. The catalyst was removed by filtration and the EtOH evaporated. The residue was distilled to give a clear oil (0.7 g, 63%): bp 106–110° (20 mm); ir (0.002 *M* CCl_4) 3450 cm^{-1} (associated OH); picrate mp 259–260°.

General Procedure for the Synthesis of *p*-Aminobenzoate Esters 1–4. A solution of the amino alcohol (0.014 mol) and TEA (0.021 mol) in 60 ml of C_6H_6 was added dropwise to a cooled solution of *p*-nitrobenzoyl chloride (0.014 mol). The mixture was refluxed for 24 hr, cooled, and extracted with 10% HCl (3 × 50 ml). The acid extracts were combined, made basic with K_2CO_3, and extracted with $CHCl_3$ (3 × 50 ml). The $CHCl_3$ was combined, dried ($MgSO_4$), and evaporated to yield a solid which was taken up in 100 ml of EtOH and added to a Parr flask. The solution was hydrogenated (3.15 kg/cm^2) over 0.2 g of 10% Pd/C for 12 hr and filtered through Celite and the solvent was evaporated to yield an orange solid. The solid was recrystallized from the indicated solvent (Table I) to yield the desired *p*-aminobenzoate ester.

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Notes

Relative Potency of (−)- and (±)-Salbutamol on Guinea Pig Tracheal Tissue†

Clifford J. Hawkins* and Gregory T. Klease

Department of Chemistry, University of Queensland, St. Lucia, Australia 4067.

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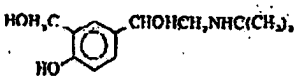
The two enantiomers of the various asymmetric β-sympathomimetic drugs are usually found to have significantly different potencies. Studies with guinea pig tracheal tissue have shown that, where the absolute configuration is known, the *R* isomer is the more active and the racemate's activity lies between those of the two enantiomers. Recently, however, it was reported that racemic salbutamol (1) was 1.5 times as active as the more active (laevo) of the two enantiomers.[1,‡]



1

This result is unique for this type of drug interaction and warranted further investigation especially as salbutamol's marked β_2 selectivity[2] has made it an important bronchodilator for the treatment of asthma.

This paper describes the results of relaxation studies with the isomers of salbutamol using guinea pig tracheal chains. Each tissue was tested by cumulative drug–response tests using adrenaline prior to study with salbutamol. The results are presented in Figure 1. The mean log ED_{50} values with their associated standard errors are as follows: isomer with $[\alpha]^{20}D$ −32.2°, −7.8 ± 0.06 (96.9 ± 4.2); (±), −7.61 ± 0.04 (101.2 ± 5.5); isomer with $[\alpha]^{20}D$ +30.8°, −7.50 ± 0.03 (98.9 ± 3.7). The mean slopes of the log dose–response curves with their standard errors are presented in parentheses. As the (+) isomer was not fully resolved, it would have somewhat less activity than that indicated by the above ED_{50}

†This investigation was supported by the Asthma Foundation of Queensland and the Australian Research Grants Committee.

‡Hartley and Middlemiss in the text of their paper[1] considered the two to be approximately equipotent.

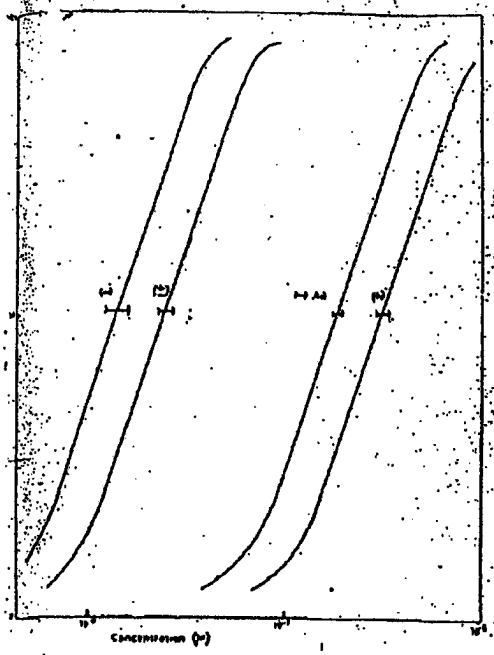


Figure 1. The mean log dose–response lines for (–), (±), and (+)-salbutamol and (–)-adrenaline. Doses are given as molar concentration in the tissue bath. The error bars represent the standard error of the mean log ED_{50}'s.

value due to contributions from the more active (–) isomer. These results, in contrast to those of Hartley and Middlemiss,[2] show that the (–) isomer is significantly more active than the racemic drug, in agreement with the general finding that a racemic drug's activity lies between those of the two enantiomers.[3,4] From a comparison of the response curves for a set of tissues, it was found that (–)- and (±)-salbutamol are 12.6 and 5.6 times, respectively, more active than (–)-adrenaline.

The technique used in the present investigation, which is a standard method for studying the relaxation of smooth muscle,[5] differs from that used by Hartley and Middlemiss.[2] Their method, which was developed in their own laboratory,[6] is an intraluminal pressure technique. The two techniques employed might be expected to give small differences in the absolute values of the ED_{50}'s but the different techniques should not yield such large variations in the relative potencies of the isomers as observed.

Experimental Section

Melting points were observed on a Büchi oil-bath melting point apparatus and microchemical analyses were performed by the Australian Microanalytical Service, Melbourne, Australia. Optical rotations were measured with a Perkin-Elmer Model 141 automatic polarimeter in H_2O at 20°. The compounds gave satisfactory uv and ir spectral data obtained with a Cary 14 and a Perkin-Elmer 225 instrument respectively.

Resolution of 2-*tert*-Butylamino-1-(4'-hydroxy-3'-hydroxymethylphenyl)ethanol (Salbutamol). To a warm solution of racemic salbutamol[8] (0.8 g, 0.0034 mol) in dilute H_2SO_4 (4 ml) was added (+)-$Na[CoEDTA]\cdot 4H_2O$ (0.76 g, 0.0008 mol), $[\alpha]^{20}_{546}$ +890° (*c* 0.5, H_2O), which was prepared from the resolved potassium salt.[7] The precipitated $BaSO_4$ was filtered off and the diastereoisomer (1.2 g) obtained by the addition of EtOH and Et_2O to the solution while cooling in ice.

(–)-2-*tert*-Butylamino-1-(4'-hydroxy-3'-hydroxymethylphenyl)-ethanol Hydrochloride Monohydrate. To the diastereoisomer (1.0 g) in H_2O (4 ml) was added BaCl, (0.22 g). The (+)-$Na[CoEDTA]\cdot 4H_2O$ was recovered by the addition of EtOH and Et_2O while cooling in ice. (–)-Salbutamol was precipitated as the HCl salt from the oil formed on evaporation of the filtrate at reduced pressure. The recrystallized product yielded 0.24 g, $[\alpha]^{20}D$ –32.2° (*c* 0.10, H_2O). The compound changed crystalline form at 175° and decomposed over the range 185–195°. *Anal.* ($C_{13}H_{22}NO_3Cl$) C, H, N.

(+)-2-*tert*-Butylamino-1-(4'-hydroxy-3'-hydroxymethylphenyl)-ethanol Hydrochloride Monohydrate. A HCl salt of (+)-salbutamol was prepared from the oil obtained on reducing the volume of the filtrate remaining after diastereoisomer removal. The recrystallized product yielded 0.15 g, $[\alpha]^{20}D$ +30.8° (*c* 0.10, H_2O). The compound changed crystalline form at 175° and decomposed over the range 185–195°. *Anal.* ($C_{13}H_{22}NO_3Cl$) C, H, N.

Relaxation Studies. The drugs were tested by a cumulative dose method on guinea pig tracheal chain at a tension of 300 mg in Krebs physiological salt solution. Linear regression lines were obtained by a least-squares method. Mean log ED_{50} and the standard error of the mean were found for each drug and tested at the 10% significance level for differences between the drugs using a student's *t* test. The mean log dose–response curves were obtained from approximately 20 tissue experiments for each drug. The tissue responses were recorded on a Hewlett-Packard 680M recorder using a Sanborn FTA-1-1 microforce transducer with a Sanborn Model 311A amplifier.

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[8] Kindly supplied by Allen and Hanburys Ltd., England.
[9] EDTA is ethylenediaminetetraacetate.

A Synthesis of Noformycin

Guy D. Diana

Sterling-Winthrop Research Institute, Rensselaer, New York 12144. *Received December 11, 1972*

Noformycin (5) was isolated from a culture of *Nocardia formica* and was identified as the active constituent of this microorganism.[1-3] This material was unusual in that it exhibited a wide range of antimicrobial activity. Of particular interest was its *in vivo* activity in mice against swine influenza and SK poliomyelitis. Subsequent to its isolation and identification, noformycin was tested against a wide variety of plant and animal viruses[4-9] and found to possess very potent activity. However, this material appeared to possess considerable toxicity, which was confirmed in our laboratories.

In view of the broad spectrum of activity, we became interested in synthesizing homologs of noformycin with the expectation of reducing toxicity while retaining activity. Specifically, we were interested in developing a versatile synthesis which would adapt itself to a variety of transformations. A detailed synthesis of noformycin itself has not been published although it has been reported that the synthetic racemic material possesses half the activity of the isomer obtained from the culture.[2] Consequently, we wish to report a facile synthesis of both racemic and (+)-noformycin which

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STUDIES ON THE EFFECTS OF ENANTIOMERS OF SOTERENOL, TRIMETOQUINOL AND SALBUTAMOL ON *BETA* ADRENERGIC RECEPTORS OF ISOLATED GUINEA-PIG ATRIA AND TRACHEA[1]

C. K. BUCKNER AND P. ABEL

School of Pharmacy, The University of Wisconsin, Madison, Wisconsin

Accepted for publication January 14, 1974

ABSTRACT

BUCKNER, C. K. AND P. ABEL: Studies on the effects of enantiomers of soterenol, trimetoquinol and salbutamol on *beta* adrenergic receptors of isolated guinea-pig atria and trachea. J. Pharmacol. Exp. Ther. 189: 616–625, 1974.

Beta adrenergic receptors of guinea-pig atria and trachea were investigated with enantiomers of tissue selective agonists: soterenol, trimetoquinol and salbutamol. If the receptors of these two tissues are different, the ratio of potencies between enantiomers of the agonists should reflect the unique asymmetries of the receptors. From atria and trachea, enantiomeric potency differences (in log units) are: for soterenol, 1.86 and 2.19; for trimetoquinol, 1.61 and 1.56 and for salbutamol, 1.86 and 2.19, respectively. From both tissues, the values for each pair of isomers are identical. Analysis of tissue selectivity from potency values reveals that (−)-isomers of soterenol, trimetoquinol and salbutamol are, respectively, 3.3-, 9- and 24-fold more potent in trachea than atria. However, when compared to (−)-isoproterenol on a relative potency basis in each tissue, only salbutamol is shown to have any degree of selectivity for trachea. When relative activities are compared from atria, soterenol and salbutamol appear as "partial agonists" while trimetoquinol produces about 90% of the maximum effect of isoproterenol. All agonists produce the same maximum effects in trachea. In atria, sotalol blocks the effects of the isomers of soterenol to a greater extent than it blocks the effects of isoproterenol and the isomers of trimetoquinol. It is suggested that factors other than ligand binding modes on the receptor could account for these observations. The data support previous suggestions that agonist binding sites on *beta* receptors of guinea-pig atria and trachea may be similar. Tissue selectivity of agonists may reflect different requirements for access to receptors or intrinsic activities between atria and trachea.

The hypothesis that there are at least two types of *beta* adrenergic receptors was advanced to explain different orders of potencies of agonists and antagonists observed between several tissues (Furchgott, 1967; Lands *et al.*, 1967a,b). The subclassifications of *beta* receptors have been supported by observations that some newly synthesized *beta* receptor agonists exhibit marked *in vivo* and *in vitro* tissue selectivity (Brittain *et al.*, 1970; Farmer *et al.*, 1970a,b). In general, the new tissue-selective agonists were shown to be more potent in relaxing bronchial and uterine smooth muscle than in producing positive cardiac

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Send reprint requests to: C. K. Buckner, School of Pharmacy, University of Wisconsin, 425 N. Charter St., Madison, Wisc. 53706.

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chronotropic or inotropic effects. Responses in the different types of tissue have been attributed to activation of so-called "*beta*-2" and "*beta*-1" receptors, respectively.

Even though tissue selectivity is clinically desired, factors other than qualitative differences in binding sites on the receptors may contribute to the phenomenon. For example, emphasizing the importance of changes in physicochemical properties with chemical structure, Buckner and Patil (1971) demonstrated that, under proper experimental conditions, the stereochemical selectivity for interaction of enantiomers of catecholamine agonists and competitive antagonists with *beta* receptors of guinea-pig atria and trachea was similar. This procedure minimized the problem of different barriers for access to the receptors by comparing potencies of compounds with the same physicochemical properties. Additionally, similar isomeric potency differences for a given pair of enantiomers suggested that the ligand binding sites on *beta* receptors of these two tissues may actually be of a similar type.

An additional problem in analysis of selectivity arises from observations that some tissue-selective agonists do not produce the same maximum mechanical effects as catecholamines in heart tissue, but appear to be "full agonists" only in smooth muscle (Farmer *et al.*, 1970a; present data). Therefore, tissue selectivity exerted by *beta* receptor agonists is not necessarily related to affinity for the receptor site, but may involve the ability to produce a response.

The availability of resolved isomers of some newer, tissue-selective *beta* receptor agonists prompted us to investigate the effects of these compounds on guinea-pig atria and trachea in light of the previous suggestion that observed tissue selectivity may not be due to different types of *beta* receptor binding sites.

Methods

Albino, female guinea pigs (O'Brien Farms, Madison, Wisc.), weighing 300 to 500 g, were killed by a sharp blow to the back of the head. The tissues were removed, trimmed of excess tissue and suspended in water-jacketed (37–38°C) 10-ml tissue baths containing a physiologic salt solution of the following composition: NaCl, 118 mM; KCl, 4.7 mM; $CaCl_2 \cdot 2H_2O$, 2.5 mM; $MgCl_2 \cdot 6H_2O$, 0.5 mM; $NaH_2PO_4 \cdot H_2O$, 1 mM; $NaHCO_3$, 25 mM; and glucose, 11 mM. The tissue baths and stock salt solution were aerated with a mixture of oxygen (95%) and carbon dioxide (5%). Mechanical responses were recorded on a Grass model 5 or model 79B polygraph *via* force-displacement transducers (FT-03).

Cumulative dose-response effects of the agonists were obtained by increasing the concentrations by a factor of about 3 while the previous dose remained in contact with the tissue (van Rossum, 1963). Each concentration was added only after the effects of the previous concentration reached maximum and remained constant. Final maximum responses were taken to be the effects occurring when a 3-fold increase in agonist concentration failed to further elicit a response. The time required to obtain complete dose-response effects varied with the agonist employed (see "Results"). All other compounds were added to the bath in a volume of 0.1 ml and allowed to interact with the tissue for fixed periods of time.

Isolated right atria. Spontaneous atrial contractions were recorded together with atrial rate which was monitored with Grass model 7P4D tachographs to aid in determining when maximum responses occurred after a given concentration of agonist. The amount of tension exerted on each atrium was the maximum needed to obtain a pen deflection of about 0.5 cm/beat at the highest preamplifier sensitivity without recording background noise. Each tissue was allowed to equilibrate for 1 hour prior to addition of any drug and washings were made at 15-minute intervals during this period. For construction of dose-response curves, the initial rate (beats per minute) was taken as that occurring just prior to beginning cumulative drug addition.

Isolated tracheal strips. Trachea were cut in spiral fashion, each turn separated by 3 to 4 cartilage segments (Constantine, 1965). Each strip was approximately halved and each half mounted in a tissue bath. Resting tension was adjusted to 5 g and maintained at that level during equilibration and drug incubation periods. Strips were allowed to equilibrate for 2 hours prior to addition of any drug and washings were made at 15-minute intervals during this period. Relaxation produced by *beta* receptor agonists was studied after partial contraction with carbachol, 3×10^{-7} M. As previously determined, this concentration produces a degree of contraction representing approximately 30% of the maximum capable of being produced by this agonist. The contraction reaches maximum in 10 to 15 minutes and remains constant for at least 1 hour. In order to keep drug contact periods constant, cumulative addition of *beta* receptor agonists was begun 15 minutes after addition of carbachol to the bath.

Experimental protocol. Because of the long duration of action of most of the agonists examined, only one cumulative dose-response curve

was obtained from a single tissue. Usually, both the (−)- and (+)-isomer of a single compound were tested on two tissues examined simultaneously. After maximum responses were obtained with each isomer of the selective agonists, (−)-isoproterenol, 10^{-4} M final bath concentration, was added and the effect produced by this treatment was taken as the maximum possible response which could be elicited through activation of the *beta* receptors. In experiments where cumulative dose-response curves were obtained with (−)-isoproterenol only, similar treatment neither increased nor decreased the already existing maximum response.

Prior to obtaining agonist-induced effects, tissues were exposed to varying pretreatments in order to impede processes which could influence observed effects of the agonists (Furchgott, 1967, 1970). In most experiments, phentolamine, 10^{-6} M, was added 40 minutes before dose-response effects were obtained in order to block *alpha* adrenergic receptors. Although this treatment decreases the spontaneous atrial rate, it does not markedly alter potencies of *beta* receptor agonists or interfere with the establishment of *beta* receptor blockade in this tissue (Krell and Patil, 1969; Buckner and Patil, 1971). The effects of the catecholamines, isoproterenol and trimetoquinol, were determined in the presence of tropolone, 10^{-4} M (30-minute contact), to inhibit the enzyme catechol-O-methyltransferase.

In some experiments, the effect of phenoxybenzamine, 10^{-6} M, on agonist-induced responses was examined by exposing the tissues to this compound for 30 minutes, followed by seven complete changes of the bath with fresh physiologic salt solution during the next 15-minute period. Fifteen minutes after the final wash, cumulative addition of a *beta* receptor agonist was begun in atria or carbachol was added to tracheal strips. Since release of endogenous norepinephrine, as produced by phenoxybenzamine (Furchgott, 1966), may influence observed effects of direct-acting agonists (Trendelenburg, 1968), tissues used in these experiments were taken from guinea pigs which had been pretreated with reserpine (5 mg/kg i.p.) 16 to 24 hours previously. In addition to irreversible *alpha* adrenergic receptor blockade (Triggle, 1965), phenoxybenzamine also blocks the adrenergic neuronal membrane uptake mechanism (Furchgott, 1966) as well as the extraneuronal uptake process and, hence, the influence of catechol-O-methyltransferase on externally applied catecholamines (Eisenfeld *et al.*, 1967).

Competitive antagonism of the effects of *beta* receptor agonists was produced by exposing the tissues to (−)-sotalol, 3×10^{-6} M, for 1 hour prior to obtaining cumulative dose-response effects of the agonists. Control dose-response curves were obtained from the paired tracheal strips or simultaneously examined right atria.

Potencies of the enantiomers are expressed as negative log molar ED50 values when responses produced by each concentration of agonist were calculated as a percentage of the final maximum response elicited by that isomer. Potency differences between enantiomers were obtained by subtracting negative log ED50 values. Because of a limited supply of (+)-salbutamol, final maximum responses in atria could not be obtained from this agonist. Therefore, atrial responses produced by each concentration of the isomers of salbutamol were calculated as a percentage of the final maximum response elicited by subsequent addition of (−)-isoproterenol. The potency difference between enantiomers of salbutamol in atria was determined from approximately parallel portions of the dose-response curves (20% of the isoproterenol-induced maximum).

Standard errors of the mean were calculated for all samples and 95% confidence intervals (C.I.) for potency differences between enantiomers.

Chemical structures of the newer agonists used in this study are shown in figure 1. All drug solutions were prepared on the day of each experiment and were kept refrigerated until shortly before use. Dilutions of the agonists were made from 10^{-2} M refrigerated stock solutions prepared in 0.9% saline with 0.05% sodium metabisulfite. Other drugs were prepared in 0.9% saline and molar strengths are expressed in terms of final bath concentrations.

The following drugs were used: (−)- and (+)-1-(3′,4′,5′-trimethoxybenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline. HCl. (trimetoquinol); (−)-, (+)- and (±)-2′-hydroxy-5′-(1-hydroxy-2-isopropylaminoethyl)methanesulfonanilide

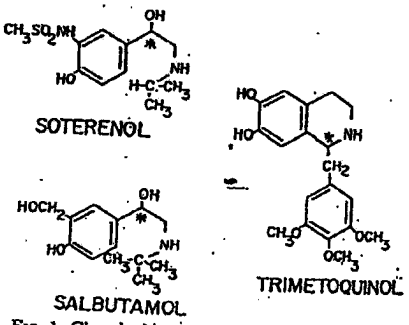

FIG. 1. Chemical structures of tissue selective *beta* receptor agonists used in the present experiments. Asterisk denotes position of the asymmetric carbon atom.

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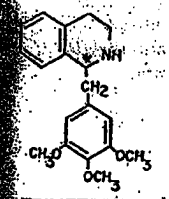

TRIMETOQUINOL

of tissue selective
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HCl (soterenol); (—)- and (+)-2-*t*-butylamino-1-(4-hydroxy-3-hydroxymethyl)phenylethanol acetate monomethanolate (salbutamol); (—)-4-(2-isopropylamino-1-hydroxyethyl) methanesulfonanilide HCl (sotalol, MJ 1999); (—)-isoproterenol-(+)-bitartrate dihydrate; carbachol chloride (Aldrich Chemical Company, Inc., Milwaukee, Wisc.); tropolone (Aldrich); phentolamine HCl (Ciba Pharmaceutical Company, Summit, N.J.); phenoxybenzamine HCl (Smith Kline and French Laboratories, Philadelphia, Pa.) and reserpine (Serpasil, Ciba). The signs (—) and (+) refer to the direction of rotation of polarized light, *levo* and *dextro*, respectively. The sign (±) refers to the racemic mixture. The same samples of isomers of the agonists were used for the entire study.

Specific rotations of the isomers of trimetoquinol and soterenol, dissolved in ethanol, were determined by optical rotatory dispersion using a Cary 60 spectropolarimeter. Calculated specific rotations from the plain dispersion curves for (—)- and (+)-trimetoquinol were —111.8 (307.5 nm) and +101.4 (307.5 nm) and for (—)- and (+)-soterenol, —197 (297.5 nm) and +218.2 (297.5 nm), respectively. These values indicate the similar degree of resolution of both isomers of the same compound.

Results

Potencies of enantiomers of selective agonists. Dose-response curves obtained from cumulative administration of the optical isomers of soterenol to isolated atria and trachea are shown in figure 2. Potency differences between the isomers are indicated by the numbers between the horizontal arrows. Data from these and other isomers are summarized in table 1.

Even though potencies of single isomers may differ as much as 24-fold (for salbutamol) between atria and trachea, for a given pair of isomers, the stereoselectivity for production of responses in the two tissues is the same. The maximum difference in enantiomeric potency ratio between the tissues is about 2-fold (0.33 log unit).

Combined reserpine and phenoxybenzamine pretreatment did not change the potency difference observed between the isomers of soterenol in either tissue (table 1). However, from both tissues, these treatments resulted in parallel shifts to the left of the dose-response curves for

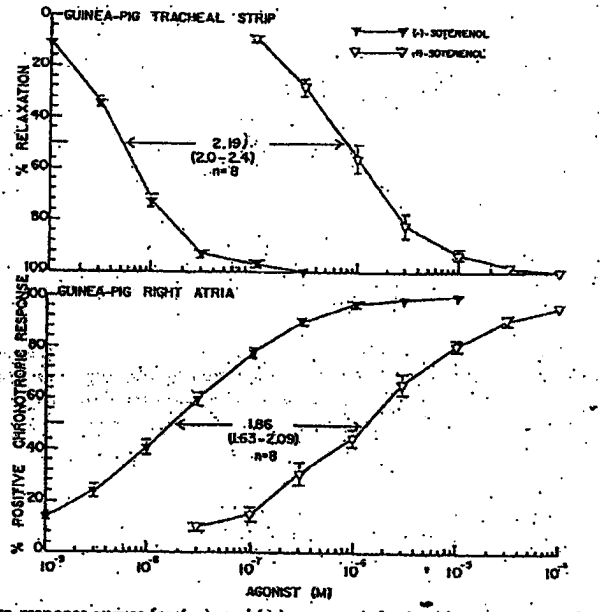

FIG. 2. Log dose-response curves for (—)- and (+)-soterenol obtained in atria and trachea taken from normal guinea pigs. Numbers between the horizontal arrows connecting the curves are enantiomeric potency differences in log units with 95% C.I. in parentheses. *n*, number of observations. All curves were obtained in the presence of phentolamine. Vertical lines indicate S.E.M.

TABLE 1

Effects of beta adrenergic receptor agonists on guinea-pig atria and tracheal strips

Agonist	Isolated Guinea-Pig Right Atria				Isolated Guinea-Pig Tracheal Strip			
	−log molar ED[b] with S.E.M.	% maximum[c] with S.E.M.	n[d]	Enantiomeric potency difference[e] (95% C.L.)	−log molar ED50 with S.E.M.	% maximum[c]	n[d]	Enantiomeric potency difference[e] (95% C.L.)
Normal tissue[a]								
(−)-Isoproterenol	8.70 ± 0.06		8		9.13 ± 0.07		8	
(−)-Soterenol	7.78 ± 0.07	65 ± 3	8	1.86	8.30 ± 0.03	100	8	2.19
(+)-Soterenol	5.92 ± 0.09	44 ± 2	8	(1.63–2.09)	6.10 ± 0.04	100	8	(2.0–2.4)
(±)-Soterenol	7.29 ± 0.07	60 ± 2	10		8.11 ± 0.04	100	6	
(−)-Trimetoquinol	8.08 ± 0.05	91 ± 1	8	1.61	9.63 ± 0.09	100	9	1.56
(+)-Trimetoquinol	7.07 ± 0.07	88 ± 1	8	(1.43–1.79)	8.08 ± 0.06	100	9	(1.33–1.79)
(−)-Salbutamol	7.70 ± 0.13	73 ± 3	6	2.29	8.44 ± 0.08	100	6	2.46
(+)-Salbutamol	5.42 ± 0.06		6	(1.97–2.60)	5.97 ± 0.07	100	6	(2.23–2.70)
Reserpine-pretreated tissue[f]								
(−)-Isoproterenol					9.26 ± 0.06		6	
(−)-Soterenol	8.13 ± 0.08	69 ± 2	9	1.85	8.99 ± 0.05	100	7	2.25
(+)-Soterenol	6.28 ± 0.06	54 ± 4	9	(1.64–2.07)	6.74 ± 0.11	100	6	(1.99–2.52)

[a] Tissues were exposed to tropolone and phentolamine. Tropolone was not used with isomers of soterenol and salbutamol. See "Methods."

[b] Negative log of the concentration of each agonist required to produce 50% of its own maximum effect. For the isomers of salbutamol, values represent negative log of the concentrations required to produce 20% of the maximum effects of (−)-isoproterenol.

[c] Maximum effect of each agonist calculated as a percentage of the maximum response produced by (−)-isoproterenol. See "Methods."

[d] *n*, number of observations.

[e] Enantiomeric potency difference = [(−log ED50 of (−)-isomer) − (−log ED50 of (+)-isomer)]. Values for salbutamol in atria calculated using concentrations required to produce 20% of the maximum effects of (−)-isoproterenol.

[f] Tissues were exposed to phenoxybenzamine and washed. See "Methods."

each isomer. Slight potentiation of the effects of isoproterenol was also observed in trachea. Slight alteration of responses to soterenol by phenoxybenzamine suggests that phenoxybenzamine-sensitive adrenergic neuronal or extraneuronal accumulation may play a small role in determining the tissue distribution of this agonist.

Data from untreated atria show that the various procedures do not markedly alter the potency difference between isomers of soterenol in this tissue. In the absence of any pretreatment of atria, −log molar ED50 values for (−)- and (+)-soterenol were 8.07 ± 0.13 (S.E.M.; n = 10) and 5.94 ± 0.22 (n = 9) while maximum effects were 65 ± 1 and 54 ± 3% of the maximum effects produced by isoproterenol, respectively.

Results from (±)-soterenol are included in table 1 as a means of comparison with the resolved isomers. Potencies of the racemate are 2 to 3 times less than those of the (−)-isomer. In atria, the maximum positive chronotropic effect produced by the racemate is also slightly less than that produced by the (−) form.

Responses produced by the isomers of selective agonists developed more slowly than those produced by isoproterenol. In both tissues, maximum effects to individual concentrations of isoproterenol usually occurred within 3 to 6 minutes after addition to the bath. This time interval was 7 to 12 minutes for the isomers of soterenol and salbutamol and 12 to 15 minutes for the isomers of trimetoquinol.

Regardless of the difficulties involved in interpretations, when potencies (−log molar ED50 values) of the (−)-isomers of the selective agonists are compared with those of (−)-isoproterenol (fig. 3), relative differences between the tis-

tracheal strips

Guinea-Pig Tracheal Strip

% maximum	n	Enantiomeric potency difference (95% C.I.)
	8	
100	8	2.19
100	8	(2.0–2.4)
100	6	
100	9	1.56
100	9	(1.33–1.79)
100	6	2.46
100	6	(2.23–2.70)
	6	
100	7	2.25
100	6	(1.99–2.52)

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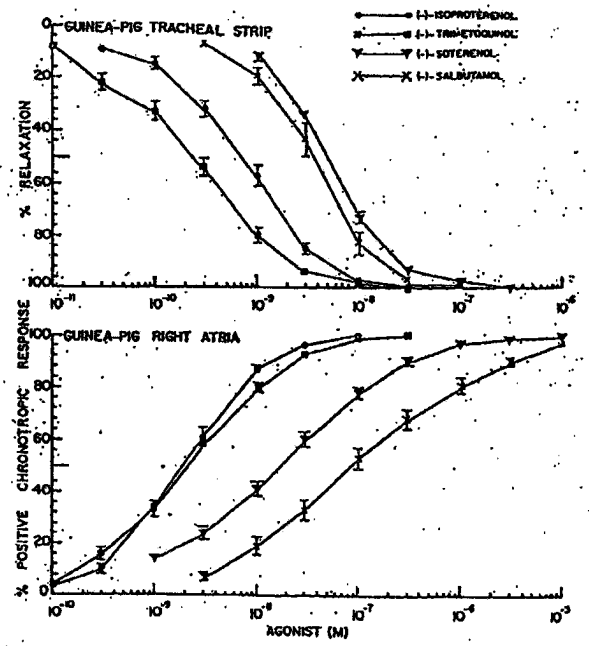

FIG. 3. Log dose-response curves for (−)-isomers of isoproterenol, soterenol, trimetoquinol and salbutamol obtained in atria and trachea taken from normal guinea pigs. See "Methods" for details on drug incubations. Percent response for each agonist concentration was calculated as a percentage of the maximum response produced by that agonist. Vertical lines indicate S.E.M.

sues are apparent only in the case of salbutamol. (−)-Salbutamol is 44 and 5 times, while (−)-soterenol is 3.5 and 7 times less potent than (−)-isoproterenol in atria and trachea, respectively. The (−) form of trimetoquinol is equally potent in atria and 3 times as potent in trachea as (−)-isoproterenol.

However, as illustrated in figure 4, these agonists are better classified as "partial agonists" in the heart since slopes of the dose-response curves and maximum positive chronotropic effects are less than those of isoproterenol. If "activities" are compared, *i.e.*, concentrations required to produce 50% of the maximum effect of isoproterenol, salbutamol is 140 times and soterenol 46 times less active than isoproterenol in atria. Trimetoquinol is approximately equal in activity to isoproterenol since these agonists produce similar maximum responses.

Antagonism by sotalol. The effects of (−)-sotalol, 3×10^{-6} M, on responses to the isomers of soterenol and (+)-isoproterenol are shown in table 2. As previously demonstrated (Buckner and Patil, 1971), sotalol is a more potent *beta* receptor antagonist in trachea. Furthermore, in trachea, sotalol produces comparable degrees of antagonism of the effects of all agonists examined. However, sotalol is selective in blocking effects of the isomers of soterenol in atria. In experiments on atria, the shifts of trimetoquinol dose-response curves produced by sotalol were (in log units) 1.05 ± 0.16 ($n = 3$) against the (−)-isomer and 0.98 ± 0.09 ($n = 3$) against the (+)-isomer. The antagonism exerted by sotalol in both tissues was presumed to be competitive in all cases since the dose-response curves were shifted to the right in parallel fashion.

Discussion

A crucial experimental criterion in the differentiation and identification of adrenergic receptors is stereochemical selectivity (Patil, 1969). Under proper experimental conditions, similar receptor types should exhibit similar stereoselec-

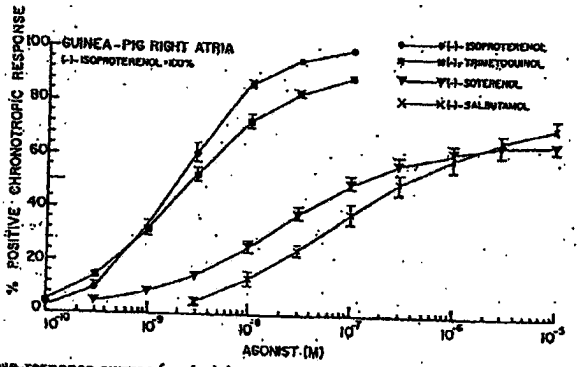

FIG. 1. Log dose-response curves for (−)-isomers of isoproterenol, soterenol, trimetoquinol and salbutamol obtained in atria taken from normal guinea pigs. See "Methods" for details on drug incubations. Percent response for each agonist concentration was calculated as a percentage of the final maximum response produced by addition of (−)-isoproterenol to each tissue. Vertical lines indicate S.E.M.

TABLE 2

Antagonism of effects of beta adrenergic receptor agonists by (−)-sotalol in guinea-pig atria and tracheal strips

Agonist	Isolated Guinea-Pig Right Atria[a]		Isolated Guinea-Pig Tracheal Strip[a]	
	Log shift with S.E.M.[b]	n[c]	Log shift with S.E.M.[b]	n[c]
(−)-Isoproterenol	0.99 ± 0.05	7	1.74 ± 0.03[d]	5
(−)-Soterenol	1.39 ± 0.11	5	1.75 ± 0.03[d]	5
(+)-Soterenol	1.32 ± 0.10	7	1.70 ± 0.09	6

[a] Tissues were exposed to tropolone and phentolamine. Tropolone was not used with isomers of soterenol. See "Methods."
[b] Log shift = (−log molar ED50 without antagonist) − (−log molar ED50 with antagonist). In atrial experiments, log shift of dose-response curves for (−)- and (+)-soterenol was determined at 30 and 20%, respectively, of the maximum response produced by isoproterenol. Concentration of antagonist was 3×10^{-6} M.
[c] n, number of paired or unpaired observations.
[d] Values taken from Buckner *et al.* (1971).

tive interactions with agonists and antagonists. Like optical isomers of classical catecholamine agonists and competitive antagonists, isomers of newer, selective *beta* receptor agonists interact with *beta* receptors of guinea-pig atria and trachea in very similar fashion. In other words, potency differences between the enantiomers are similar in the two tissues regardless of the position of the dose-response curves along the log dose axis. For example, even though (−)-trimetoquinol is 10 times more potent in trachea than atria, the (+)-isomer exhibits the same degree of tissue selectivity. The present observations from potencies of enantiomers of selective agonists support the suggestion that *beta* receptors of guinea-pig atria and trachea may be similar (Buckner and Patil, 1971).

The agonist action of trimetoquinol adds new dimensions to structure-activity investigations of *beta* receptors. Whereas other agonists possess a center of asymmetry at the β carbon atom of the phenethanolamine structure, trimetoquinol is a cyclized derivative with no substitution at the site corresponding to the β hydroxyl. These differences suggest that it combines with additional receptor regions and interacts at an alternative asymmetric site on the receptor. The similar potency differences for trimetoquinol in atria and trachea suggest the similarity of these sites and strengthen the suggested similar nature of the receptor sites in the two tissues.

A major assumption associated with the use of optical isomers to differentiate receptors is that responses to the lesser active isomer are not entirely due to contamination of the sample by the more active isomer. Even though similar specific rotations from optical rotatory dispersion measurements (see "Methods") suggest similar degrees of resolution of the isomers of soterenol and trimetoquinol, in the absence of a pure standard the degree of impurities in each sample can not be determined. However, absolute stereochemical purity, although desirable, is not essential in the pharmacologic experiments provided that the same chemical samples are used in all studies and that the less active (+)-isomers

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do not have zero potency. At least one indirect line of evidence suggests that (+)-isomers of adrenergic drugs possess their own effects. In most cases, it has been shown that (+)-isomers are equal in potency to the corresponding desoxy derivatives (Patil *et al.*, 1970). This relationship is predicted by the Easson and Stedman hypothesis (1933) that (+)-isomers act as if the alcoholic OH were missing since this group, by being oriented away from the receptor, would not contribute to the affinity of the molecule for the receptor. The desoxy derivative of soterenol has equal potency to (+)-soterenol in guinea-pig trachea and rat uterus (Dr. G. R. McKinney, personal communication) and therefore, conforms to this hypothesis. This allows the assumption that the effects produced by (+)-soterenol are elicited mainly by that isomer. The desoxy derivative of salbutamol has not been tested and the hypothesis may not now be applied to trimetoquinol.

Farmer *et al.* (1970a) and Brittain *et al.* (1970) reported that racemic forms of soterenol, salbutamol and trimetoquinol were, respectively, 3.3, 500, and >10,000 times less potent in guinea-pig atria and 5, 5 and 2 times less potent in guinea-pig trachea than isoproterenol. Regardless of the manner in which the values are obtained, our data do not reveal such largely different relative potencies for salbutamol and trimetoquinol between the two tissues. In our experiments, salbutamol exhibited greater selectivity than trimetoquinol and soterenol which, under most experimental conditions, were minimally selective for trachea. More recently, Brittain *et al.* (1973) reported a difference in potency between isomers of salbutamol in isolated guinea-pig trachea which is approximately one-fourth the value obtained in our experiments. Furthermore, they were unable to demonstrate appreciable positive chronotropic effects in guinea-pig right atria using either isomer. In both tissues, effective concentrations of the isomers appear to be about 100 times greater than in our experiments. As outlined by Furchgott (1967), one of the experimental conditions which must be satisfied in analyzing drug-receptor interactions is that sufficient time be allowed for steady-state responses to develop after addition of each drug concentration. The selective agonists have slower rates of onset of action than isoproterenol and, unless this factor is considered in studying pharmacologic effects, a highly potent agonist could appear less potent. In addition, *alpha* adrenergic receptor activation by these compounds could interfere with observed *beta* receptor potency and this factor can be eliminated by addition of an *alpha* receptor antagonist.

Even under appropriate experimental conditions of the present study, salbutamol, soterenol and, to a lesser degree, trimetoquinol could be classified as "partial agonists" in atria. This could account for some of the reported selectivity when "activities" rather than "potencies" are evaluated. Since activity measures the concentration required to produce 50% of the maximum response to a standard agonist, this parameter for a partial agonist like salbutamol would be determined in the upper portion of the dose-response curve where the slope is diminishing. The potency of an agonist is measured in the steep portion of the dose-response curve (at 50% of the maximum produced by that agonist) and is expected to more accurately reflect receptor binding. The lesser relative activity of the agonist-induced effects in only atrial preparation could be related to 1) different degrees of receptor reserve (Ariens, 1964). 2) greater desensitization during cumulative drug addition, 3) non-competitive action beyond receptor activation and or 4) different degrees of access to receptor sites.

Regardless of the interpretation, decreased ability of an agonist to produce a response in one tissue as opposed to another does not provide compelling evidence that the receptor binding sites in the two tissues are different. Although similar stereo-chemical selectivity for agonist activity in two tissues is not absolute proof that the binding sites are the same, it is one of the criteria which must be used in receptor classification.

Effects of agonists acting on the same receptor should be blocked to the same extent by a competitive antagonist (Arunlakshana and Schild, 1959). However, it has been argued that different degrees of blockade by the same antagonist in two tissues does not necessarily suggest a difference of receptor type in those tissues (Buckner and Patil, 1971; Buckner and Christopherson, 1974). Hence, there are alternative means of explaining selectivity for trachea exerted by sotalol in our experiments. However, selective blockade by sotalol of effects of the isomers of soterenol in guinea-pig atria is not explained on

the basis of these considerations. Carlsson *et al.* (1972) demonstrated a similar phenomenon in cat atria and suggested the possibility that there is an array of binding modes on the receptor such that structurally varied agonists would not necessarily interact with the same configuration of the receptor. According to this model, an antagonist could also selectively bind to one of these sites. The close structural similarity between sotalol and soterenol suggests that they combine with similar sites. However, are these the exact sites with which isoproterenol interacts? In trachea, sotalol does not exert selective blockade of the different agonists. On the basis of enantiomeric potency differences reported for several agonists and antagonists, the *beta* receptors of guinea-pig atria and trachea may be similar. Therefore, an explanation for selective blockade of agonists in only atria should be sought in events not involving differences in the specific receptors. For example, a partial agonist like soterenol also acts as a competitive antagonist (Ariens, 1964; Raper and Malta, 1973) and may produce additive antagonism during cumulative drug addition. Alternatively, isoproterenol may have additional means of producing responses which could not be blocked by a specific receptor or antagonist. An interesting possibility is that inhibition of phosphodiesterase by catecholamines may contribute to mechanical produced by these compounds (Goren and Rosen, 1972; Hitchcock, 1973). The several possibilities should be explored in quantitative fashion before making conclusions about ligand binding modes on the receptor.

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NEW UTILITY PATENT APPLICATION TRANSMITTAL
(Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No: 0701.027F

Total Pages in this Submission

TO THE ASSISTANT COMMISSIONER FOR PATENTS
Box Patent Application
Washington, D.C. 20231

Transmitted herewith for filing under 35 U.S.C. 111(a) and 37 C.F.R. 1.53(b) is a new utility patent application for the invention entitled:

METHOD FOR TREATING ASTHMA USING OPTICALLY PURE R(-) ALBUTEROL

and invented by:

Timothy J. Barberich and James W. Young

If a CONTINUATION APPLICATION, *check appropriate box and supply the requisite information:*

☒ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No.: 08/691,604

Enclosed are:

Application Elements

1. ☒ Filing fee as calculated and transmitted as described below
2. ☒ Specification having 10 pages and including the following:
   - a. ☒ Descriptive Title of the Invention
   - b. ☐ Cross References to Related Applications *(if applicable)*
   - c. ☐ Statement Regarding Federally-sponsored Research/Development *(if applicable)*
   - d. ☐ Reference to Microfiche Appendix *(if applicable)*
   - e. ☒ Background of the Invention
   - f. ☒ Brief Summary of the Invention
   - g. ☐ Brief Description of the Drawings *(if drawings filed)*
   - h. ☒ Detailed Description
   - i. ☒ Claim(s) as Classified Below
   - j. ☒ Abstract of the Disclosure
3. ☐ Drawing(s) *(when necessary as prescribed by 35 USC 113)*
   - a. ☐ Formal
   - b. ☐ Informal

   Number of Sheets _____

NEW UTILITY PATENT APPLICATION TRANSMITTAL
(Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No.
0701.027F

Total Pages in this Submission

Application Elements (Continued)

4. ☒ Oath or Declaration
   a. ☐ Newly executed *(original or copy)* ☐ Unexecuted
   b. ☒ Copy from a prior application (37 CFR 1.63(d)) *(for continuation/divisional application only)*
   c. ☐ With Power of Attorney ☐ Without Power of Attorney

5. ☒ Incorporation By Reference *(usable if Box 4b is checked)*
   The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

6. ☐ Computer Program in Microfiche

7. ☐ Genetic Sequence Submission *(if applicable, all must be included)*
   a. ☐ Paper Copy
   b. ☐ Computer Readable Copy
   c. ☐ Statement Verifying Identical Paper and Computer Readable Copy

Accompanying Application Parts

8. ☐ Assignment Papers *(cover sheet & documents)*

9. ☐ 37 CFR(B) Statement *(when there is an assignee)*

10. ☐ English Translation Document *(if applicable)*

11. ☒ Information Disclosure Statement/PTO-1449 ☐ Copies of IDS Citations

12. ☒ Preliminary Amendment

13. ☒ Acknowledgment postcard

14. ☒ Certificate of Mailing
    ☐ First Class ☒ Express Mail *(Specify Label No.):* EL042394445US

15. ☐ Certified Copy of Priority Document(s) *(if foreign priority is claimed)*

16. ☐ Small Entity Statement(s) - Specify Number of Statements Submitted: _____

NEW UTILITY PATENT APPLICATION TRANSMITTAL
(Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No. 0701.027F

Total Pages in this Submission

Accompanying Application Parts (Continued)

17. ☒ Additional Enclosures *(please identify below):*

Three (3) Terminal Disclaimers and fee ($165) therefor
Copy of Associate Power of Attorney

Fee Calculation and Transmittal

CLAIMS AS FILED

For	#Filed	#Allowed	#Extra	Rate	Fee
Total Claims	12	- 20 =	0	x $11.00	$0.00
Indep. Claims	3	- 3 =	0	x $41.00	$0.00
Multiple Dependent Claims (check if applicable) ☐					$0.00
				BASIC FEE	$395.00
OTHER FEE *(specify purpose)*					$0.00
				TOTAL FILING FEE	$395.00

☒ A check in the amount of $560.00 to cover the filing fee and Terminal Disclaimers is enclosed.
☒ The Commissioner is hereby authorized to charge and credit Deposit Account No. 08-1935 as described below. A duplicate copy of this sheet is enclosed.
- ☐ Charge the amount of as filing fee.
- ☒ Credit any overpayment.
- ☒ Charge any additional filing fees required under 37 C.F.R. 1.16 and 1.17.
- ☐ Charge the issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance, pursuant to 37 C.F.R. 1.311(b).

Dated: April 21, 1998

Signature

Philip E. Hansen
Reg. No. 32,700

Heslin & Rothenberg, P.C.
5 Columbia Circle
Albany, NY 12203

ADDRESS ALL FUTURE CORRESPONDENCE TO:

PhilipE. Hansen
HESLIN & ROTHENBERG, P.C.
5 Columbia Circle
Albany, New York 12203